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(21) International Application Number: PCT/AU98/00898 (22) International Filing Date: 28 October 1998 (28.10.98) (30) Priority Data: PP 0062 28 October 1997 (28.10.97) AU PP 0812 9 December 1997 (09.12.97) AU (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 09/181,336 (CIP) Filed on 27 October 1998 (27.10.98) (71) Applicant (for all designated States except US): THE AUSTRALIAN NATIONAL UNIVERSITY [AU/AU]; Acton, ACT 2601 (AU). (72) Inventors; and (75) Inventors/Applicants (for US only): FREEMAN, Craig, Geoffrey [AU/AU]; 31 Woollum Place, Rivett, ACT 2611 (AU). HULETT, Mark, Darren [AU/AU]; 14 Teague Street, Cook, ACT 2614 (AU). PARISH, Christopher, Richard [AU/AU]; 62 Vasey Crescent, Campbell, ACT 2601 (AU). HAMDORF, Brenton, James [AU/AU]; 25 Rowe Place, Swinger Hill, ACT 2606 (AU).		(74) Agents: SLATTERY, John, Michael et al.; Davies Collison Cave, 1 Little Collins Street, Melbourne, VIC 3000 (AU). (81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: ISOLATED NUCLEIC ACID MOLECULE ENCODING MAMMALIAN ENDOGLUCURONIDASE AND USES THEREFOR (57) Abstract <p>The present invention relates to isolated or recombinant mammalian endoglucuronidase enzymes, polypeptides and peptides, in particular human, murine and rat heparanases, genetic sequences encoding same and uses therefor, for example in the determination and characterisation of chemical compounds, proteins, polypeptides, small molecules and macromolecules capable of inhibiting metastasis, angiogenesis, angioplasty-induced restenosis, atherosclerosis, inflammation, promote wound healing and otherwise modulate physiological processes involving heparanase cleavage of heparan sulphate. The invention further relates to a method of altering, modifying or otherwise modulating the level of expression of mammalian heparanase in a cell. A further aspect of the invention relates to immunoreactive molecules capable of binding to and/or inhibiting mammalian heparanase, in particular monoclonal antibodies. A still further aspect of the invention contemplates the use of heparanase as an agent to promote the processes of wound healing.</p>		

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ISOLATED NUCLEIC ACID MOLECULE ENCODING MAMMALIAN ENDOGLUCURONIDASE AND USES THEREFOR

FIELD OF THE INVENTION

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The invention relates to isolated or recombinant mammalian endoglucuronidase enzymes, polypeptides and peptides, in particular human platelet heparanase, genetic sequences encoding the same and uses therefor, for example in the determination and characterisation of chemical compounds, proteins, polypeptides, small molecules and macromolecules capable
10 of inhibiting metastasis, angiogenesis, angioplasty-induced restenosis, atherosclerosis, inflammation, promote wound healing and otherwise modulate physiological processes involving heparanase cleavage of heparan sulphate. The invention further relates to a method of altering, modifying or otherwise modulating the level of expression of mammalian heparanase in a cell. A further aspect of the invention relates to immunoreactive molecules
15 capable of binding to and/or inhibiting mammalian heparanase, in particular monoclonal antibodies. A still further aspect of the invention contemplates the use of heparanase as an agent to inhibit the processes of neovascularisation.

GENERAL

20 Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description.

This specification contains nucleotide and amino acid sequence information prepared using the programme PatentIn Version 2.0, presented herein after the bibliography. Each
25 nucleotide or amino acid sequence is identified in the sequence listing by the numeric indicator <210> followed by the sequence identifier (e.g. <210> 1, <210> 2, etc). The length, type of sequence (DNA, protein (PRT), etc) and source organism for each nucleotide or amino acid sequence are indicated by information provided in the numeric indicator fields <211>, <212> and <213>, respectively. Nucleotide and amino acid sequences referred
30 to in the specification are defined by the information provided in numeric indicator field

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<400> followed by the sequence identifier (eg. <400> 1, <400> 2, etc).

The designation of nucleotide residues referred to herein are those recommended by the IUPAC-IUB Biochemical Nomenclature Commission, wherein A represents Adenine, C
5 represents Cytosine, G represents Guanine, T represents thymine, Y represents a pyrimidine residue, R represents a purine residue, M represents Adenine or Cytosine, K represents Guanine or Thymine, S represents Guanine or Cytosine, W represents Adenine or Thymine, H represents a nucleotide other than Guanine, B represents a nucleotide other than Adenine, V represents a nucleotide other than Thymine, D represents a nucleotide other than Cytosine
10 and N represents any nucleotide residue.

The designations for amino acid residues referred to herein are set forth in Table I.

As used herein the term "derived from" shall be taken to indicate that a specified integer may
15 be obtained from a particular source albeit not necessarily directly from that source.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated step or element or integer or group of steps or elements or integers but not the
20 exclusion of any other step or element or integer or group of elements or integers.

BACKGROUND OF THE INVENTION

Tissue invasion by blood-borne malignant tumour cells and leukocytes involves their adhesion to the luminal surface of the vascular endothelium, passage through the vascular endothelial cell layer and the subsequent degradation of the underlying basal lamina and extracellular matrix (ECM) with a battery of secreted and/or cell surface protease and glycosidase activities (Nakajima *et al.*, 1983; Schmitt *et al.*, 1992; Vlodavsky *et al.*, 1992).

Studies have shown that while the initial entrapment of metastatic tumour cells by the capillary endothelium is platelet-independent, platelet aggregation which occurs shortly thereafter can lead to platelet activation and degranulation, resulting in gap formation and retraction of endothelial cells, exposing the underlying basement membrane to adhesion by the tumour cells (Tanaka *et al.*, 1986; Crissman *et al.*, 1985; Yahalom *et al.*, 1985).

The basal lamina and underlying connective tissue stroma consist predominantly of a complex network of fibronectin, laminin, collagen type IV and vitronectin, each of which interact with heparan sulphate (HS) side chains of heparan sulphate proteoglycans (HSPG) embedded within the matrix (Yurchenco and Schittny, 1990).

HS chains generally consist of clusters of sulphated disaccharide units (predominantly N-sulphated glucosamine linked 1-4 to α -L-iduronic acid residues) separated by lowly or non-sulphated regions (predominantly disaccharide units of N-acetylated glucosamine linked 1-4 to β -D-glucuronic acid) (Turnbull and Gallagher, 1990; 1991).

In work leading up to the present invention, the inventors sought to isolate and characterise enzymes, proteins, polypeptides and peptides which are capable of cleaving the HS side chains of HSPG embedded in the matrix and genetic sequences encoding same. The genetic sequences thus derived provide a means for assisting the disassembly of the ECM and facilitating cell migration, when expressed at the matrix site or transported thereto.

The genetic sequences of the present invention further provide the means for developing a wide range of therapeutic and prophylactic pharmaceutical compounds to inhibit metastasis, neovascularisation, angiogenesis, angioplasty-induced restenosis, atherosclerotic plaque formation and inflammation and/or to promote wound healing, amongst others.

5

SUMMARY OF THE INVENTION

One aspect of the invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides encoding or complementary to a sequence encoding a polypeptide capable of
10 hydrolysing glycosidic bonds in HS.

A second aspect of the invention provides an isolated nucleic acid molecule comprising a sequence of nucleotides which encodes or is complementary to a sequence which encodes a mammalian endoglucuronidase polypeptide, in particular heparanase or fragment or derivative
15 thereof. More particularly, the mammalian endoglucuronidase polypeptide comprises an amino acid sequence as set forth in any one or more of <400>1-11 or <400>13 or <400>15 or <400>17 or <400>19 or <400>23 or is at least 40% identical thereto.

A further aspect of the invention provides an isolated nucleic acid molecule which is at least
20 40% identical to the nucleotide sequence set forth in any one of <400>12 or <400>14 or <400>16 or <400>18 or a homologue, analogue or derivative thereof, or a complementary sequence thereto.

A still further aspect of the present invention provides a genetic construct which expresses
25 a recombinant endoglucuronidase activity, in particular heparanase activity or an active site thereof.

Another aspect of the invention provides a recombinant mammalian endoglucuronidase polypeptide, in particular heparanase or fragment or derivative thereof.

30

Still yet another aspect of the invention contemplates a method of identifying a modulator of heparanase activity, said method comprising assaying recombinant heparanase activity in the presence of a potential modulator and comparing said activity to the activity of recombinant heparanase in the absence of said potential modulator.

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A further aspect of the invention contemplates an inhibitor of a mammalian endoglucuronidase polypeptide, in particular a mammalian heparanase. The inhibitor molecules encompassed by the invention are particularly useful as inhibitors of metastasis, angiogenesis, wound healing, angioplasty-induced restenosis, arteriosclerosis, atherosclerosis, inflammation or other
10 physiological or medical condition wherein heparanase activity is elevated.

In still yet another aspect of the invention there is contemplated the use of recombinant heparanase or an active fragment or derivative thereof to inhibit neovascularisation and its associated processes involved in the regulation of tissue development, inflammation, wound
15 healing and tumour metastasis.

The recombinant polypeptides of the invention are also useful in the sequencing of sulphated molecules such as HSPG and heparan sulphate molecules or to assist in the determination of the structure of sulphated proteoglycans, sulphated oligosaccharides and heparan sulphate
20 molecules, wherein said recombinant polypeptide is used to cleave the heparan sulphate moiety therefrom.

A further aspect of the invention provides an immunologically interactive molecule which is capable of binding to the recombinant endoglucuronidase polypeptide of the invention, in
25 particular an antibody molecule which is capable of binding to and/or inhibiting the catalytic activity of a heparanase polypeptide. The antibody molecules of the invention are particularly useful in the diagnosis of heparanase expression in biological samples, particularly where patients are suspected of having a condition associated with elevated heparanase expression such as cancer, metastasis, angiogenesis, angioplasty-induced restenosis, atherosclerosis or
30 inflammation, amongst others.

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A further aspect of the invention provides a recombinant endoglucuronidase polypeptide, in particular a recombinant heparanase polypeptide or an immunologically interactive homologue, analogue or derivative thereof for use as a "standard" in the diagnosis of heparanase expression of biological samples, particularly in diagnostic assays of patient-
5 derived samples such as serum wherein the patients are suspected of having a condition associated with elevated heparanase expression, such as those listed *supra*.

A still further aspect of the invention contemplates a method of diagnosing elevated heparanase expression in a human or animal subject said method comprising contacting an
10 antibody molecule which is capable of binding to a heparanase polypeptide with a biological sample such as serum or isolated cells derived from said subject for a time and under conditions sufficient for an antibody:antigen complex to form and then detecting and/or quantifying the complex thus formed. Quantification according to this aspect of the invention is performed using a standard protein which comprises recombinant heparanase or a
15 homologue, analogue or derivative thereof.

A still further aspect of the invention contemplates a method of diagnosing elevated heparanase expression in a human or animal subject, said method comprising contacting a biological sample which comprises mRNA encoding heparanase derived from said subject or
20 an isolate mRNA sample encoding heparanase derived from said subject with an isolated nucleic acid molecule which comprises a nucleotide sequence capable of binding to said mRNA encoding heparanase for a time and under conditions sufficient for hybridisation to occur and then detecting and/or quantifying said hybridisation.

25 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a photographic representation of purified human platelet heparanase and of deglycosylated purified human platelet heparanase following SDS-PAGE. Purified platelet heparanase was reduced with dithioerythritol and electrophoresed on a 10% polyacrylamide
30 gel and stained with Coomassie Brilliant Blue R250. Lane 1, Mr standards (phosphorylase

b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soya-bean trypsin inhibitor (20 kDa) and α -lactalbumin (14 kDa)); lane 2, human platelet heparanase and lane 3, membrane-associated human platelet heparanase. Human platelet heparanase was incubated with a) no enzyme (lane 4), b) N-glycosidase F (lane 5) and 5 c) N-glycanase F, O-glycosidase and neuraminidase (lane 6).

Figure 2 is a graphical representation of the expression vector pcDNA3 (Invitrogen) showing the location of the cytomegalovirus IE promoter (P CMV), BGH terminator (BGH pA), SV40 origin of replication (SV40 ori), neomycin resistance gene (Neomycin), SV40 terminator 10 (SV40 pA), bacterial origin of replication (ColE1) and ampicillin resistance gene (Ampicillin). The endoglucuronidase-coding sequences of the present invention are inserted in to the multiple cloning site (HindIII....ApaI) which is flanked by the T7 and SP6 promoter sequences.

15 **Figure 3** is a copy of a photographic representation of a Northern blot hybridisation of mRNAs derived from non-immune heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas tissues, following hybridisation with radioactively-labelled full-length human heparanase cDNA set forth in <400>12. Tissue sources are indicated at the top of each lane. Size markers (kb) are indicated at the left of the Figure.

20

Figure 4 is a copy of a photographic representation of a Northern blot hybridisation of mRNAs derived from immune spleen, lymph node, thymus, peripheral blood (PB) leukocyte, bone marrow and fetal liver tissues, following hybridisation with radioactively-labelled full-length human heparanase cDNA set forth in <400>12. Tissue sources are indicated at the 25 top of each lane. Size markers (kb) are indicated at the left of the Figure.

Figure 5 is a copy of a photographic representation of a Genomic Southern blot hybridisation showing the gene organisation and copy number of the human heparanase gene. Genomic DNA from two individuals (lanes marked 1 and 2) was digested with the restriction enzymes 30 *EcoRI* (lanes 1 and 2), *BamHI* (lanes 3 and 4), *HindIII* (lanes 5 and 6) or *PstI* (lanes 7 and

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8), separated by electrophoresis on a 1% (w/v) agarose gel, transferred to nylon membrane and hybridised to a radioactively-labelled full-length human heparanase cDNA clone (<400>12). Enzymes used and source of DNA are indicated at the top of the lanes. Size markers (kb) are indicated at the left of the Figure. The arrow at the right-hand side of the

5 Figure indicates the position of a polymorphic 1.4 kb *Pst*I fragment that is present in individual 2 but not individual 1.

Figure 6 is a copy of a schematic representation showing an alignment of the human (<400>13), murine (<400>17) and rat (<400>19) heparanase amino acid sequences.

10 The sequences of the human (hu.hep), murine (mu.hep) and rat (rat.hep) heparanase polypeptides were aligned using the PILEUP programme at the Computer Genetics Group (Devereaux *et al*, 1984). Identical amino acids are boxed. Numbers refer to the amino acid positions for each of the sequences shown in the Figure.

15 Figure 7 is a copy of a graphical representation showing the ELISA titres of antisera obtained using a 15-amino acid-long peptide derived from residues 423 to 437 of human heparanase (<400>23) and conjugated to KLH (filled bars) compared to antisera obtained using platelet-derived heparanase (diagonal cross-hatched bars) or compared to preimmune serum obtained from rabbits prior to peptide-KLH immunization (horizontal cross-hatched bars). Data show

20 the optical density (y-axis) for each serum dilution tested (x-axis). Samples marked CON is a non-serum control sample.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

25 One aspect of the present invention provides an isolated nucleic acid molecule which comprises a nucleotide sequence which encodes polypeptide capable of cleaving the HS side chains of HSPG or a complementary nucleotide sequence thereto.

The term "isolated" means that a stated integer or group of integers is provided in a form

30 which is distinct from that which occurs in nature, preferably wherein one or more

contaminants have been removed.

As used herein, the term "cleaving" or similar term includes the hydrolysis of one or more glycosidic bonds of HS.

5

The nucleic acid molecule of the invention may be RNA or DNA (e.g. cDNA), single or double stranded and linear or covalently closed. The nucleic acid molecule may also be genomic DNA corresponding to the entire gene or a substantial portion thereof or to fragments and derivatives thereof. The nucleotide sequence may correspond to the naturally occurring nucleotide sequence or may contain single or multiple nucleotide substitutions, deletions or additions.

More particularly, the isolated nucleic acid molecule may be one or more of the following molecules:

- 15 (i) a classical genomic gene consisting of transcriptional and/or translational regulatory sequences and/or a coding region and/or non-translated sequences (i.e. introns, 5'- and 3'- untranslated sequences);
- (ii) mRNA or cDNA corresponding to the coding region or a part thereof or one or more exon sequences, and 5'-untranslated sequences and/or 3'- untranslated sequences of the gene;
- 20 (iii) a structural region corresponding to the coding region or a part thereof or one or more exon sequences; and/or
- (iv) a synthetic or fusion molecule encoding a functional endoglucuronidase polypeptide or heparanase polypeptide or a homologue, analogue or derivative thereof.

25

In a particularly preferred embodiment of the present invention, the isolated nucleic acid molecule is a cDNA molecule.

As used herein, the term "polypeptide" shall be taken to refer to any polymer which at least comprises amino acids including a non-enzymatically-active peptide molecule or an

30

enzymatically-active protein of enzyme molecule or alternatively, a fusion molecule. A reference to "polypeptides" shall also be taken to include both naturally-occurring molecules and recombinantly-produced molecules.

- 5 In a preferred embodiment of the present invention, the polypeptide product of the isolated nucleic acid molecule is an endoglucuronidase polypeptide or a homologue, analogue or derivative thereof.

As used herein, the term "endoglucuronidase" shall be taken to refer to any peptide,
10 polypeptide, protein or enzyme molecule which is at least capable of cleaving a sulphated disaccharide or sulphated polysaccharide from a sulphated proteoglycan molecule.

Those skilled in the art are aware that the endoglucuronidases include both heparanases and endoglycosidases, amongst others which are at least capable of hydrolysing or otherwise
15 cleaving one or more sulphated disaccharide units from proteoglycans. However, not all endoglucuronidases possess high activity on all proteoglycan substrates and some degree of substrate specificity generally occurs for enzymes within this class.

For example, murine melanoma B16 heparanase cleaves both heparin and HS albeit not at
20 equal efficiency (Graham and Underwood, 1996). On the other hand, tumour-derived heparanase is unable to degrade endothelial cell surface HSPG (Hennes *et al.*, 1988), whereas human platelets degrade both endothelial cell surface HSPG, tumour-derived HSPG, ECM-associated HSPC and other structures which are more heparin-like in structure (Hoogewerf
25 Wasteson *et al.*, 1976; Wasteson *et al.*, 1977; Gamse *et al.*, 1978), presumably *via* the heparanase activity therein.

As used herein the term "heparanase" shall be taken to refer to any peptide, polypeptide, protein or enzyme molecule which is at least capable of removing the HS side chain from
30 HSPG associated with the endothelial cell surface and/or the extracellular matrix (ECM)

and/or tumour cells and/or heparin, and includes both recombinant molecules, isolated naturally-occurring isoforms and fusion polypeptides.

Preferably, the endoglucuronidase polypeptide is heparanase, or a homologue, analogue or derivative thereof, more preferably heparanase polypeptide which is at least capable of degrading endothelial cell surface HSPG by cleaving the HS side chain(s) therefrom, even more preferably a heparanase polypeptide which is at least capable of degrading both endothelial cell surface HSPG and ECM-associated HSPG and even more preferably a heparanase polypeptide which is at least capable of cleaving endothelial cell surface HSPG, tumour-derived HSPG, ECM-associated HSPG and heparin-like HS side chains, including heparin.

As exemplified herein, the present inventors have isolated the heparanase enzyme from human platelets, determined the N-terminal amino acid sequence and amino acid sequence of tryptic peptides of the heparanase polypeptide and utilised the amino acid sequence to isolate a cDNA molecule which encodes platelet heparanase.

Accordingly, in a particularly preferred embodiment the present invention provides an isolated nucleic acid molecule which encodes or is complementary to an isolated nucleic acid molecule which encodes a heparanase polypeptide which at least comprises an amino acid sequence which is at least 40% identical to the sequence set forth in any one of <400>1-11 or <400>13 or <400>15 or <400>17 or <400>19 or <400>23.

Preferably, the percentage similarity to any one of <400>1-11 or <400>13 or <400>15 or <400>17 or <400>19 or <400>23 is at least about 60%, more preferably at least about 80%, even more preferably at least about 90%.

In determining whether or not two amino acid sequences fall within these percentage limits, those skilled in the art will be aware that it is necessary to conduct a side-by-side comparison or multiple alignment of sequences. In such comparisons or alignments, differences will arise

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in the positioning of non-identical residues, depending upon the algorithm used to perform the alignment. In the present context, reference to a percentage identity or similarity between two or more amino acid sequences shall be taken to refer to the number of identical and similar residues respectively, between said sequences as determined using any standard
5 algorithm known to those skilled in the art. For example, amino acid sequence identities or similarities may be calculated using the GAP programme and/or aligned using the PILEUP programme of the Computer Genetics Group, Inc., University Research Park, Madison, Wisconsin, United States of America (Devereaux *et al*, 1984). The GAP programme utilizes the algorithm of Needleman and Wunsch (1970) to maximise the number of identical/similar
10 residues and to minimise the number and/or length of sequence gaps in the alignment. Alternatively or in addition, wherein more than two amino acid sequences are being compared, the ClustalW programme of Thompson *et al* (1994) is used.

In an alternative embodiment, the isolated nucleic acid molecule of the invention encodes or
15 is complementary to an isolated nucleic acid molecule which encodes a heparanase polypeptide which at least comprises an amino acid sequence which is substantially identical to any one of <400>1-11 or <400>13 or <400>15 or <400>17 or <400>19 or <400>23.

As used herein, the term "substantially identical" or similar term shall be taken to include any
20 sequence which is at least about 95% identical to a stated nucleotide sequence or amino acid sequence, including any homologue, analogue or derivative of said stated nucleotide sequence or amino acid sequence.

For the purposes of nomenclature, the amino acid sequences set forth in <400>1-11 relate to the
25 amino acid sequences of tryptic peptides derived from the purified heparanase polypeptide. The complete amino acid sequence of the human heparanase polypeptide is set forth in <400>13. The amino acid sequence of a human heparanase polypeptide derivative used to produce antibodies suitable for diagnostic applications, described in Example 8, is set forth in <400>23. The complete amino acid sequence of a variant human heparanase polypeptide is set forth in
30 <400>15. The partial amino acid sequence of the murine heparanase polypeptide is set forth

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in <400>17. The Partial amino acid sequence of the rat heparanase polypeptide is set forth in <400>19.

In the present context, "homologues" of an endoglucuronidase or heparanase polypeptide
5 refer to those polypeptides, enzymes or proteins which have a similar activity to the human heparanase polypeptide and are at least about 40% identical thereto, notwithstanding any amino acid substitutions, additions or deletions. A homologue may be isolated or derived from the same species as the heparanase polypeptide exemplified herein or from a different species.

10

Furthermore, the amino acids of a homologous polypeptide may be replaced by other amino acids having similar properties, for example hydrophobicity, hydrophilicity, hydrophobic moment, charge or antigenicity, and so on.

15

"Analogues" encompass functional and non-functional polypeptides which have at least about 40% amino acid sequence identity to human heparanase notwithstanding the occurrence of any non-naturally occurring amino acid analogues therein.

20 The term "derivative" in relation to endoglucuronidase or heparanase polypeptide described herein shall be taken to refer hereinafter to mutants, parts or fragments derived from the heparanase polypeptide which may or may not possess the activity of the functional protein. Derivatives include modified peptides in which ligands are attached to one or more of the amino acid residues contained therein, such as carbohydrates, enzymes, proteins, polypeptides
25 or reporter molecules such as radionuclides or fluorescent compounds. Glycosylated, fluorescent, acylated or alkylated forms of the subject peptides are particularly contemplated by the present invention. Additionally, derivatives of heparanase which comprise fragments or parts of an amino acid sequence disclosed herein are within the scope of the invention, as are homopolymers or heteropolymers comprising two or more copies of the subject
30 polypeptides. Procedures for derivatizing peptides are well-known in the art.

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Substitutions encompass amino acid alterations in which an amino acid of the base polypeptide (i.e. heparanase) is replaced with a different naturally-occurring or a non-conventional amino acid residue. Such substitutions may be classified as "conservative", in which case an amino acid residue contained in the base polypeptide is replaced with another
5 naturally-occurring amino acid of similar character, for example Gly↔Ala, Val↔Ile↔Leu, Asp↔Glu, Lys↔Arg, Asn↔Gln or Phe↔Trp↔Tyr.

Substitutions encompassed by the present invention may also be "non-conservative", in which an amino acid residue which is present in the base polypeptide is substituted with an amino
10 acid having different properties, such as a naturally-occurring amino acid from a different group (eg. substituted a charged or hydrophobic amino acid with alanine), or alternatively, in which a naturally-occurring amino acid is substituted with a non-conventional amino acid.

15 Amino acid substitutions are typically of single residues, but may be of multiple residues, either clustered or dispersed.

Naturally-occurring amino acids include those listed in Table 1. Non-conventional amino acids encompassed by the invention include, but are not limited to those listed in Table 2.
20

Amino acid deletions will usually be of the order of about 1-10 amino acid residues, while insertions may be of any length. Deletions and insertions may be made to the N-terminus, the C-terminus or be internal deletions or insertions. Generally, insertions within the amino acid sequence will be smaller than amino-or carboxyl-terminal fusions and of the order of 1-4
25 amino acid residues.

Those skilled in the art will be aware that several means for producing homologue, analogue or derivatives of a base polypeptide are possible when provided with the isolated nucleic acid molecule which encodes said polypeptide, for example site-directed mutagenesis of DNA and
30 polymerase chain reaction utilising mutagenised oligonucleotide primers, amongst others.

Accordingly, the present invention clearly extends to any and all homologue, analogue or derivatives of the endoglucuronidase or heparanase polypeptides of the present invention.

TABLE 1

5	Amino Acid	Three-letter Abbreviation	One-letter Symbol
	Alanine	Ala	A
10	Arginine	Arg	R
	Asparagine	Asn	N
	Aspartic acid	Asp	D
	Cysteine	Cys	C
	Glutamine	Gln	Q
15	Glutamic acid	Glu	E
	Glycine	Gly	G
	Histidine	His	H
	Isoleucine	Ile	I
	Leucine	Leu	L
20	Lysine	Lys	K
	Methionine	Met	M
	Phenylalanine	Phe	F
	Proline	Pro	P
	Serine	Ser	S
25	Threonine	Thr	T
	Tryptophan	Trp	W
	Tyrosine	Tyr	Y
	Valine	Val	V
30	Any amino acid as above	Xaa	X

TABLE 2

Non-conventional amino acid	Code	Non-conventional amino acid	Code
5 α -aminobutyric acid	Abu	L-N-methylalanine	Nmala
α -amino- α -methylbutyrate	Mgab	L-N-methylarginine	Nmarg
aminocyclopropane- carboxylate	Cpro	L-N-methylasparagine	Nmasn
10 aminoisobutyric acid	Aib	L-N-methylaspartic acid	Nmasp
aminonorbornyl- carboxylate	Norb	L-N-methylcysteine	Nmcys
cyclohexylalanine	Chexa	L-N-methylglutamine	Nmgln
cyclopentylalanine	Cpen	L-N-methylglutamic acid	Nmglu
15 D-alanine	Dal	L-N-methylhistidine	Nmhis
D-arginine	Darg	L-N-methylisoleucine	Nmile
D-aspartic acid	Dasp	L-N-methylleucine	Nmleu
D-cysteine	Dcys	L-N-methyllysine	Nmlys
D-glutamine	Dgln	L-N-methylmethionine	Nmmet
20 D-glutamic acid	Dglu	L-N-methylnorleucine	Nmnle
D-histidine	Dhis	L-N-methylnorvaline	Nmnva
D-isoleucine	Dile	L-N-methylornithine	Nmorn
D-leucine	Dleu	L-N-methylphenylalanine	Nmphe
D-lysine	Dlys	L-N-methylproline	Nmpro
25 D-methionine	Dmet	L-N-methylserine	Nmser
D-ornithine	Dorn	L-N-methylthreonine	Nmthr
D-phenylalanine	Dphe	L-N-methyltryptophan	Nmtrp
D-proline	Dpro	L-N-methyltyrosine	Nmtyr
D-serine	Dser	L-N-methylvaline	Nmval
30 D-threonine	Dthr	L-N-methylethylglycine	Nmetg
		L-N-methyl-t-butylglycine	Nmtbug
		L-norleucine	Nle

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	D-tryptophan	Dtrp	L-norvaline	Nva
	D-tyrosine	Dtyr	α -methyl-aminoisobutyrate	Maib
	D-valine	Dval	α -methyl- γ -aminobutyrate	Mgabu
	D- α -methylalanine	Dmala	α -methylcyclohexylalanine	Mchexa
5	D- α -methylarginine	Dmarg	α -methylcyclopentylalanine	Mcpen
	D- α -methylasparagine	Dmasn	α -methyl- α -naphthylalanine	Manap
	D- α -methylaspartate	Dmasp	α -methylpenicillamine	Mpen
	D- α -methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
	D- α -methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
10	D- α -methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
	D- α -methylisoleucine	Dmile	N-amino- α -methylbutyrate	Nmaabu
	D- α -methyllleucine	Dmleu	α -naphthylalanine	Anap
	D- α -methyllysine	Dmlys	N-benzylglycine	Nphe
	D- α -methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
15	D- α -methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
	D- α -methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
	D- α -methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
	D- α -methylserine	Dmser	N-cyclobutylglycine	Ncbut
	D- α -methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
20	D- α -methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
	D- α -methyltyrosine	Dmty	N-cyclodecylglycine	Ncdec
	D- α -methylvaline	Dmval	N-cylcododecylglycine	Ncdod
	D-N-methylalanine	Dnmala	N-cyclooctylglycine	Ncoct
	D-N-methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
25	D-N-methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
	D-N-methylaspartate	Dnmasp	N-(2,2-diphenylethyl) glycine	Nbhm
	D-N-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl) glycine	Nbhe

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	D-N-methylglutamine	DnmglN	N-(3-guanidinopropyl) glycine	Narg
	D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
	D-N-methylhistidine	Dnmhis	N-(hydroxyethyl)glycine	Nser
5	D-N-methylisoleucine	Dnmile	N-(imidazolylethyl) glycine	Nhis
	D-N-methylleucine	Dnmleu	N-(3-indolylyethyl) glycine	Nhtrp
	D-N-methyllysine	Dnmlys	N-methyl- γ -aminobutyrate	Nmgabu
10	N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmt
	D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpn
	N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
	N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
	N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
15	N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
	D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
	D-N-methyltyrosine	Dnmtyr	N-methyl-naphthylalanine	Nmanap
	D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
	γ -aminobutyric acid	Gabu	N-(<i>p</i> -hydroxyphenyl)glycine	Nhtyr
20	L- <i>t</i> -butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
	L-ethylglycine	Etg	penicillamine	Pen
	L-homophenylalanine	Hphe	L- α -methylalanine	Mala
	L- α -methylarginine	Marg	L- α -methylasparagine	Masn
	L- α -methylaspartate	Masp	L- α -methyl- <i>t</i> -butylglycine	Mtbug
25	L- α -methylcysteine	Mcys	L-methylethylglycine	Metg
	L- α -methylglutamine	Mgln	L- α -methylglutamate	Mglu
	L- α -methylhistidine	Mhis	L- α -methylhomo phenylalanine	Mhphe
	L- α -methylisoleucine	Mile	N-(2-methylthioethyl) glycine	Nmet
30				

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L- α -methyllleucine	Mleu	L- α -methyllysine	Mlys
L- α -methylmethionine	Mmet	L- α -methylnorleucine	Mnle
L- α -methylnorvaline	Mnva	L- α -methylornithine	Morn
L- α -methylphenylalanine	Mphe	L- α -methylproline	Mpro
5 L- α -methylserine	Mser	L- α -methylthreonine	Mthr
L- α -methyltryptophan	Mtrp	L- α -methyltyrosine	Mtyr
L- α -methylvaline	Mval	L-N-methylhomo	
		phenylalanine	Nmhpe
N-(N-(2,2-diphenylethyl)		N-(N-(3,3-diphenylpropyl)	
10 carbamylmethyl)glycine	Nnbhm	carbamylmethyl)glycine	Nnbhe
1-carboxy-1-(2,2-diphenyl-			
ethylamino)cyclopropane	Nmbc		

- 15 The isolated nucleic acid molecule of the invention is preferably derived from a mammalian source, such as a human or laboratory animal such as, a mouse, rabbit or rat, amongst others. In a particularly preferred embodiment, the isolated nucleic acid molecule is derived from a human.
- 20 As used herein, the term "derived from" shall be taken to refer to the origin of an integer or group of integers from a specified source, but not to the exclusion of other possible source or sources of said integer or group of integers.

The invention clearly extends to all tissue sources of the subject nucleic acid molecule, in particular wherein the isolated nucleic acid molecule comprises genomic DNA.

Preferred tissue sources of mRNA encoding an endoglucuronidase polypeptide or heparanase polypeptide include liver, placenta, spleen, platelets, macrophages and tumour cells such as, but not limited to melanoma cells, mammary adenocarcinoma cells, colonic carcinoma cells and B16 tumour cells, amongst others.

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In a particularly preferred embodiment of the invention, the isolated nucleic acid molecule is derived from human platelets, murine spleen T-cells or rat MAT cells.

A further aspect of the present invention contemplates a nucleic acid molecule which encodes
5 or is complementary to a nucleic acid molecule which encodes, an endoglucuronidase polypeptide wherein said nucleic acid molecule is capable of hybridising under at least low stringency conditions to the nucleic acid molecule set forth in any one of <400>12 or <400>14 or <400>16 or <400>18 or a complementary strand thereto.

10 For the purposes of nomenclature, the nucleotide sequence set forth in <400>12 relates to the cDNA encoding human platelet heparanase, an endoglucuronidase enzyme encompassed by the present invention. The nucleotide sequence set forth in <400>14 relates to a variant cDNA encoding human platelet heparanase. The nucleotide sequence set forth in <400>16
15 relates to the mouse activated spleen T cell-derived partial heparanase cDNA fragment produced by PCR using the oligonucleotides designated BamHepN and mhép3. The nucleotide sequence set forth in <400>18 relates to the rat MAT cell-derived partial heparanase cDNA fragment produced by PCR using the oligonucleotides designated BamHepN and dT-Not.

20 Those skilled in the art will be aware that variants of the human platelet heparanase cDNA sequence set forth in any one of <400>12 or <400>14 or <400>16 or <400>18 may be isolated by hybridization under low stringency conditions. Such variants include any genomic sequences, cDNA sequences mRNA or other isolated nucleic acid molecules derived from humans or other mammals. Additional variants are not excluded.

25

Preferably, the nucleic acid molecule further comprises a nucleotide sequence which encodes, or is complementary to a nucleotide sequence which encodes, a heparanase polypeptide, more preferably a heparanase polypeptide having the catalytic activity described *supra*.

30 More preferably, the isolated nucleic acid molecule according to this aspect of the invention

is capable of hybridising under at least medium stringency conditions to the nucleic acid molecule set forth in any one of <400>12 or <400>14 or <400>16 or <400>18 or to a complementary strand thereof.

- 5 Even more preferably, the isolated nucleic acid molecule according to this aspect of the invention is capable of hybridising under at least high stringency conditions to the nucleic acid molecule set forth in any one of <400>12 or <400>14 or <400>16 or <400>18 or to a complementary strand thereof.
- 10 For the purposes of defining the level of stringency, a low stringency is defined herein as being a hybridisation and/or a wash carried out in 6xSSC buffer, 0.1 % (w/v) SDS at 28°C. Generally, the stringency is increased by reducing the concentration of SSC buffer, and/or increasing the concentration of SDS and/or increasing the temperature of the hybridisation and/or wash. A medium stringency comprises a hybridisation and/or a wash carried out in
- 15 0.2xSSC-2xSSC buffer, 0.1 % (w/v) SDS at 42°C to 65°C, while a high stringency comprises a hybridisation and/or a wash carried out in 0.1xSSC-0.2xSSC buffer, 0.1 % (w/v) SDS at a temperature of at least 55°C. Conditions for hybridisations and washes are well understood by one normally skilled in the art. For the purposes of further clarification only, reference to the parameters affecting hybridisation between nucleic acid molecules is found in pages
- 20 2.10.8 to 2.10.16. of Ausubel *et al.* (1987), which is herein incorporated by reference.

In an even more preferred embodiment of the invention, the isolated nucleic acid molecule further comprises a sequence of nucleotides which is at least 40% identical to at least 10 contiguous nucleotides derived from any one of <400>12 or <400>14 or <400>16 or

25 <400>18 or a complementary strand thereof.

Still more preferably, the isolated nucleic acid molecule further comprises a sequence of nucleotides which is at least 40% identical to at least 50 contiguous nucleotides derived from the sequence set forth in any one of <400>12 or <400>14 or <400>16 or <400>18

30 or a complementary strand thereof.

In determining whether or not two nucleotide sequences fall within these percentage limits, those skilled in the art will be aware that it is necessary to conduct a side-by-side comparison or multiple alignment of sequences. In such comparisons or alignments, differences may
5 arise in the positioning of non-identical residues, depending upon the algorithm used to perform the alignment. In the present context, reference to a percentage identity between two or more nucleotide sequences shall be taken to refer to the number of identical residues between said sequences as determined using any standard algorithm known to those skilled in the art. For example, nucleotide sequences may be aligned and their identity calculated
10 using the BESTFIT programme or other appropriate programme of the Computer Genetics Group, Inc., University Research Park, Madison, Wisconsin, United States of America (Devereaux *et al*, 1984).

The present invention is particularly directed to a nucleic acid molecule which is capable of
15 encoding a mammalian endoglucuronidase polypeptide, in particular mammalian heparanase polypeptide, for example human heparanase derived from platelets. The subject invention clearly contemplates additional genes to those specifically described herein which are derived from human platelets.

20 A genetic sequence which encodes or is complementary to a sequence which encodes a mammalian endoglucuronidase polypeptide such as human heparanase may correspond to the naturally occurring sequence or may differ by one or more nucleotide substitutions, deletions and/or additions. Accordingly, the present invention extends to any endoglucuronidase or heparanase genes and any functional genes, mutants, derivatives, parts, fragments,
25 homologues or analogues thereof or non-functional molecules but which are at least useful as, for example, genetic probes, or primer sequences in the enzymatic or chemical synthesis of said gene, or in the generation of immunologically interactive recombinant molecules.

In a particularly preferred embodiment, the genetic sequences of the invention exemplified
30 herein are employed to identify and isolate similar genes from other cells, tissues, or organ

types of the same or a different species, or from the cells, tissues, or organs of another mammalian species, in particular a laboratory mammal such as a rat, mouse or rabbit.

According to this embodiment, genomic DNA, or mRNA, or cDNA derived from said other
5 cells, tissues or organs with a hybridisation effective amount of a first heparanase-encoding genetic sequence comprising any one of <400>12 or <400>14 or <400>16 or <400>18 or a complementary sequence, homologue, analogue or derivative thereof derived from at least 10 contiguous nucleotides of said first sequence, and then detecting said hybridisation.

10

For the present purpose, "homologues" of a nucleotide sequence shall be taken to refer to an isolated nucleic acid molecule which is substantially the same as the nucleic acid molecule of the present invention or its complementary nucleotide sequence, notwithstanding the occurrence within said sequence, of one or more nucleotide substitutions, insertions,
15 deletions, or rearrangements.

"Analogues" of a nucleotide sequence set forth herein shall be taken to refer to an isolated nucleic acid molecule which is substantially the same as a nucleic acid molecule of the present invention or its complementary nucleotide sequence, notwithstanding the occurrence of any
20 non-nucleotide constituents not normally present in said isolated nucleic acid molecule, for example carbohydrates, radiochemicals including radionucleotides, reporter molecules such as, but not limited to DIG, alkaline phosphatase or horseradish peroxidase, amongst others.

25 "Derivatives" of a nucleotide sequence set forth herein shall be taken to refer to any isolated nucleic acid molecule which contains significant sequence similarity to said sequence or a part thereof. Generally, the nucleotide sequence of the present invention may be subjected to mutagenesis to produce single or multiple nucleotide substitutions, deletions and/or insertions.

Nucleotide insertional derivatives of the nucleotide sequence of the present invention include
30 5' and 3' terminal fusions as well as intra-sequence insertions of single or multiple

nucleotides or nucleotide analogues. Insertional nucleotide sequence variants are those in which one or more nucleotides or nucleotide analogues are introduced into a predetermined site in the nucleotide sequence of said sequence, although random insertion is also possible with suitable screening of the resulting product being performed. Deletional variants are
5 characterised by the removal of one or more nucleotides from the nucleotide sequence. Substitutional nucleotide variants are those in which at least one nucleotide in the sequence has been removed and a different nucleotide or nucleotide analogue inserted in its place.

In a particularly preferred embodiment, the heparanase-encoding genetic sequence is labelled
10 with a reporter molecule capable of giving an identifiable signal (e.g. a radioisotope such as ^{32}P or ^{35}S or a biotinylated molecule).

Preferably, the first genetic sequence comprises at least 50 contiguous nucleotides, even more preferably at least 100 contiguous nucleotides and even more preferably at least 500
15 contiguous nucleotides, derived from any one of <400> 12 or <400> 14 or <400> 16 or <400> 18 or a complementary strand, homologue, analogue or derivative thereof.

The related genetic sequence thus identified may be in a recombinant form, in a virus particle, bacteriophage particle, yeast cell, animal cell, or a plant cell.
20

An alternative method contemplated in the present invention involves hybridising two nucleic acid "primer molecules" derived from the heparanase-encoding sequence exemplified herein, to a nucleic acid "template molecule" which at least comprises a nucleotide sequence encoding a related genetic sequence or a functional part thereof, wherein the first of said primers
25 comprises contiguous nucleotides derived from any one of <400> 12 or <400> 14 or <400> 16 or <400> 18 or a homologue, analogue or derivative thereof and the second of said primers comprises contiguous nucleotides complementary to <400> 12 or <400> 14 or <400> 16 or <400> 18 or a homologue, analogue or derivative thereof, subject to the proviso that the first and second primers are not complementary to each other. Specific
30 nucleic acid molecule copies of the template molecule are amplified enzymatically in a

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polymerase chain reaction, a technique that is well known to one skilled in the art.

In a preferred embodiment, each nucleic acid primer molecule is at least 10 nucleotides in length, more preferably at least 20 nucleotides in length, even more preferably at least 30
5 nucleotides in length, still more preferably at least 40 nucleotides in length and even still more preferably at least 50 nucleotides in length.

Furthermore, the nucleic acid primer molecules consists of a combination of any of the nucleotides adenine, cytidine, guanine, thymidine, or inosine, or functional analogues or
10 derivatives thereof which are at least capable of being incorporated into a polynucleotide molecule without having an inhibitory effect on the hybridisation of said primer to the template molecule in the environment in which it is used.

Furthermore, one or both of the nucleic acid primer molecules may be contained in an
15 aqueous mixture of other nucleic acid primer molecules, for example a mixture of degenerate primer sequences which vary from each other by one or more nucleotide substitutions or deletions. Alternatively, one or both of the nucleic acid primer molecules may be in a substantially pure form.

20 The nucleic acid template molecule may be in a recombinant form, in a virus particle, bacteriophage particle, yeast cell, animal cell, or a plant cell. Preferably, the nucleic acid template molecule is derived from a human or laboratory animal species.

Those skilled in the art will be aware that there are many known variations of the basic
25 polymerase chain reaction procedure, which may be employed to isolate a related genetic sequence encoding an endoglucuronidase or heparanase polypeptide when provided with the nucleotide sequence set forth in any one of <400>12 or <400>14 or <400>16 or
<400>18. Such variations are discussed, for example, in McPherson *et al* (1991). The present invention extends to the use of all such variations in the isolation of related
30 endoglucuronidase-encoding or heparanase-encoding genetic sequences using the nucleotide

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sequences exemplified herein.

The isolated nucleic acid molecule according to any of the further embodiments may be cloned into a plasmid or bacteriophage molecule, for example to facilitate the preparation of primer molecules or hybridisation probes or for the production of recombinant gene products. Methods for the production of such recombinant plasmids, cosmids, bacteriophage molecules or other recombinant molecules are well-known to those of ordinary skill in the art and can be accomplished without undue experimentation. Accordingly, the invention further extends to any recombinant plasmid, bacteriophage, cosmid or other recombinant molecule comprising the nucleotide sequence set forth in any one of <400> 12 or <400> 14 or <400> 16 or <400> 18 or a complementary sequence, homologue, analogue or derivative thereof.

The nucleic acid molecule of the present invention is also useful for developing genetic constructs which express the endoglucuronidase polypeptide of the present invention, thereby providing for the production of the recombinant polypeptide in isolated cells or transformed tissues.

A third aspect of the present invention provides a genetic construct comprising an isolated nucleic acid molecule which encodes or is complementary to a nucleic acid molecule which encodes a mammalian endoglucuronidase polypeptide, in particular a mammalian heparanase polypeptide as described herein.

In a most preferred embodiment, the genetic construct is an expression vector.

The term "expression vector" refers to a genetic construct wherein an isolated nucleic acid molecule is provided in an expressible form by placing said molecule in operable connection with appropriate regulatory sequences such as promoters and terminators, which are required for cell-based expression to occur. In the present context, an expression vector includes genetic constructs in which an isolated nucleic acid molecule which encodes an

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endoglucuronidase or heparanase polypeptide is placed in operable connection with a suitable promoter in the sense orientation to facilitate expression of a recombinant polypeptide when the expression vector is introduced into a cell. An expression vector also encompasses genetic constructs in which the isolated nucleic acid molecule is placed in operable connection with
5 a suitable promoter in the antisense orientation to facilitate the transcription of an inhibitory nucleic acid molecule, for example an antisense molecule, ribozyme or minizyme.

Accordingly, one embodiment of the invention provides an expression vector which is useful for the production of the recombinant endoglucuronidase or heparanase polypeptide or
10 alternatively, an antisense molecule, ribozyme or minizyme, when introduced into a cell line or a virus particle and under conditions suitable for gene expression or at least transcription to occur. Such conditions will depend upon the selection of a suitable cell line and expression vector, including the selection of promoter and terminator sequences to regulate expression, and would be well-known to the person skilled in the art.

15

Reference herein to a "promoter" is to be taken in its broadest context and includes the transcriptional regulatory sequences of a classical genomic gene, including the TATA box which is required for accurate transcription initiation in a eukaryotic cell, with or without a CCAAT box sequence or alternatively, the Pribnow box required for accurate expression in
20 prokaryotic cells.

The promoter may include further regulatory elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or in a tissue-specific manner. Preferred promoters may contain additional
25 copies of one or more specific regulatory elements, to further enhance expression and/or to alter the spatial expression and/or temporal expression pattern. For example, regulatory elements which confer copper inducibility may be placed adjacent to a heterologous promoter sequence driving expression of a structural gene or recombinase gene, thereby conferring copper inducibility on the expression of said gene.

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In the present context, the term "promoter" is also used to describe a synthetic or fusion molecule, or derivative which confers, activates or enhances expression in a cell.

A promoter is usually, but not necessarily, positioned upstream or 5', of a structural gene, the expression of which it regulates. Furthermore, the regulatory elements comprising a promoter are usually positioned within 2 kb of the start site of transcription of the gene.

Placing a gene or isolated nucleic acid molecule operably under the control of a promoter sequence means positioning said gene or isolated nucleic acid molecule such that its expression is controlled by the promoter sequence. Promoters are generally positioned 5' (upstream) to the genes that they control. In the construction of heterologous promoter/structural gene combinations it is generally preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting, i.e., the gene from which the promoter is derived. As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the preferred positioning of a regulatory sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting, i.e., the genes from which it is derived. Again, as is known in the art, some variation in this distance can also occur.

20

Those skilled in the art will recognise that the choice of promoter will depend upon the nature of the cell being transformed and when expression of the recombinase, structural gene or other gene contained in the genetic construct of the invention is required. Furthermore, it is well-known in the art that the promoter sequence used in the expression vector will also vary depending upon the level of expression required and whether expression is intended to be constitutive or regulated.

For expression in eukaryotic cells, the genetic construct generally comprises, in addition to the nucleic acid molecule of the invention, a promoter and optionally other regulatory sequences designed to facilitate expression of said nucleic acid molecule. The promoter may

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be derived from a genomic clone encoding a mammalian endoglucuronidase such as heparanase or alternatively, it may be a heterologous promoter derived from another genetic source. Promoter sequences suitable for expression of genes in eukaryotic cells are well-known in the art.

5

Suitable promoters for use in eukaryotic expression vectors include those capable of regulating expression in mammalian cells, insect cells such as Sf9 (*Spodoptera frugiperda*) cells, yeast cells and plant cells. Preferred promoters for expression in eukaryotic cells include the polyhedron promoter, the SV40 early promoter and the cytomegalovirus (CMV-10 IE) promoter, amongst others.

Wherein the expression vector is intended for the production of recombinant protein, the promoter is further selected such that it is capable of regulating expression in a cell which is capable of performing any post-translational modification to the polypeptide which may be 15 required for the subject recombinant polypeptide to be functional, such as N-linked glycosylation. Cells suitable for such purposes may be readily determined by those skilled in the art. By way of exemplification, Chinese hamster ovary (CHO) cells may be employed to carry out the N-terminal glycosylation and signal sequence cleavage of a recombinant polypeptide produced therein. Alternatively, a baculovirus expression vector such as the 20 pFastBac vector supplied by GibcoBRL may be used to express recombinant endoglucuronidase polypeptides in Sf9 (*Spodoptera frugiperda*) cells, following standard protocols.

Numerous expression vectors suitable for the present purpose have been described and are 25 readily available. In a particularly preferred embodiment, the expression vector is based upon the pcDNA3 vector distributed by Medos Company Pty Ltd, Victoria, Australia which comprises the CMV promoter and BGH terminator sequences for regulating expression of the recombinant endoglucuronidase polypeptide of the invention in a eukaryotic cell, when isolated nucleic acid sequences encoding same are inserted, in the sense orientation relative 30 to the CMV promoter, into the multiple cloning site of said vector. For the purposes of

exemplification only, a map of the pcDNA3 vector is provided in Figure 2.

Examples of eukaryotic cells contemplated herein to be suitable for expression include mammalian, yeast, insect, plant cells or cell lines such as COS, VERO, HeLa, mouse C127,
5 Chinese hamster ovary (CHO), WI-38, baby hamster kidney (BHK), MDCK or Sf9 (insect) cell lines. Such cell lines are readily available to those skilled in the art.

The prerequisite for expression in prokaryotic cells such as *Escherichia coli* is the use of a strong promoter with an effective ribosome binding site. Typical promoters suitable for
10 expression in bacterial cells such as *E. coli* include, but are not limited to, the *lacZ* promoter, temperature-sensitive λ_L or λ_R promoters, T7 promoter or the IPTG-inducible *tac* promoter. A number of other vector systems for expressing the nucleic acid molecule of the invention in *E. coli* are well-known in the art and are described for example in Ausubel *et al* (1987).

15 Numerous vectors having suitable promoter sequences for expression in bacteria have been described, such as for example, pKC30 (λ_L : Shimatake and Rosenberg, 1981), pKK173-3 (*tac*: Amann and Brosius, 1985), pET-3 (T7: Studier and Moffat, 1986) or the pQE series of expression vectors (Qiagen, CA), amongst others.

20 Suitable prokaryotic cells include corynebacterium, salmonella, *Escherichia coli*, *Bacillus* sp. and *Pseudomonas* sp, amongst others. Bacterial strains which are suitable for the present purpose are well-known in the relevant art (Ausubel *et al*, 1987).

The term "terminator" refers to a DNA sequence at the end of a transcriptional unit which
25 signals termination of transcription, in particular 3'-non-translated DNA sequences. In the case of terminators for transcription in prokaryotic cells, the terminator generally includes a polyadenylation signal, which facilitates the addition of polyadenylate sequences to the 3' end of a primary transcript. They may be isolated from bacteria, fungi, viruses, animals and/or plants. Terminators active in eukaryotic and prokaryotic cells are known and
30 described in the literature. Examples of terminators particularly suitable for use in the genetic

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constructs of the present invention include the BGH polyadenylation sequence.

The genetic constructs described herein may further comprise genetic sequences corresponding to a bacterial origin of replication and/or a selectable marker gene such as an
5 antibiotic-resistance gene, suitable for the maintenance and replication of said genetic construct in a prokaryotic or eukaryotic cell, tissue or organism. Such sequences are well-known in the art.

Selectable marker genes include genes which when expressed are capable of conferring
10 resistance on a cell to a compound which would, absent expression of said selectable marker gene, prevent or slow cell proliferation or result in cell death. Preferred selectable marker genes contemplated herein include, but are not limited to antibiotic-resistance genes such as those conferring resistance to ampicillin, Claforan, gentamycin, G-418, hygromycin, rifampicin, kanamycin, neomycin, spectinomycin, tetracycline or a derivative or related
15 compound thereof or any other compound which may be toxic to a cell.

The origin of replication or a selectable marker gene will be spatially-separated from those genetic sequences which encode the recombinant endoglucuronidase or heparanase polypeptide.
20

In one particularly preferred embodiment of the present invention, the expression vector is intended for production of a recombinant mammalian endoglucuronidase or heparanase polypeptide. Accordingly, in such embodiments, it is essential that the nucleotide sequence which encodes said polypeptide be placed in the sense orientation relative to the promoter
25 sequence to which it is operably connected.

Preferably, the recombinant polypeptide which is produced is functional. Those skilled in the art will realise that notwithstanding that the nucleic acid molecule of the invention is derived from a mammalian cell, it may be possible to express a functional recombinant polypeptide
30 encoded therefor in either a prokaryotic or eukaryotic cell line. Appropriate cell lines for

expression of a functional recombinant endoglucuronidase polypeptide may readily be determined without undue experimentation. Preferably however, the recombinant polypeptide is expressed using a eukaryotic cell line, more preferably a mammalian cell line such as any one of the cell lines described *supra*.

5

Preferably, the recombinant polypeptide produced comprises an amino acid sequence which is at least 40% identical to any one or more of <400> 1-11 or <400> 13 or <400> 15 or <400> 17 or <400> 19 or <400> 23 or a homologue, analogue or derivative thereof, more preferably including any post-translational modifications thereto, in particular one or
10 more glycosylated amino acids.

In an alternative embodiment, the recombinant endoglucuronidase or heparanase polypeptide is produced as an "in-frame" fusion polypeptide with a second polypeptide, for example a detectable reporter polypeptide such as β -galactosidase, β -glucuronidase, luciferase or other
15 enzyme or a hapten peptide such as a poly-lysine or poly-histidine or other polypeptide molecule.

By "in-frame" means that a nucleotide sequence which encodes a first polypeptide is placed (i.e. cloned or ligated) in the same open reading frame adjacent to a nucleotide sequence
20 which encodes a second polypeptide with no intervening stop codons there between, such that when the ligated nucleic acid molecule is expressed, a single fusion polypeptide is produced which comprises a sequence of amino acids corresponding to the summation of the individual amino acid sequences of the first and second polypeptides.

25 In order to produce a fusion polypeptide, the nucleic acid molecule which encodes the endoglucuronidase or heparanase polypeptide or a homologue, analogue or derivative thereof is cloned adjacent to a second nucleic acid molecule encoding the second polypeptide, optionally separated by a spacer nucleic acid molecule which encodes one or more amino acids (eg: poly-lysine or poly histidine, amongst others), such that the first coding region and
30 the second coding region are in the same open reading frame, with no intervening stop codons

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between the two coding regions. When translated, the polypeptide thus produced comprises a fusion between the polypeptide products of the first and second coding regions. Wherein a spacer nucleic acid molecule is utilised in the genetic construct, it may be desirable for said spacer to at least encode an amino acid sequence which is cleavable to assist in separation of
5 the fused polypeptide products of the first and second coding regions, for example a thrombin cleavage site.

A genetic construct which encodes a fusion polypeptide further comprises at least one start codon and one stop codon, capable of being recognised by the cell's translational machinery
10 in which expression is intended.

Preferably, a genetic construct which encodes a fusion polypeptide may be further modified to include a genetic sequence which encodes a targeting signal placed in-frame with the coding region of the endoglucuronidase-encoding or heparanase-encoding nucleotide
15 sequence, to target the expressed recombinant endoglucuronidase polypeptide or heparanase polypeptide to the extracellular matrix. More preferably, the genetic sequence encoding targeting signal is placed in-frame at the 5'-terminus or the 3'-terminus, but most preferably at the 5'-terminus, of the coding region of the nucleotide sequence which encodes the endoglucuronidase or heparanase polypeptide.

20

Methods for the production of a fusion polypeptide are well-known to those skilled in the art.

In order to produce the recombinant endoglucuronidase or heparanase polypeptide of the invention, the expression vector described herein is introduced into an appropriate cell line
25 by any means known to those skilled in the art, for example by electroporation, calcium chloride transformation or PEG fusion, amongst others, to produce a transformed cell or transfected cell. The transformed or transfected cell is subsequently incubated for a time and under conditions sufficient for expression of the recombinant polypeptide encoded by the genetic construct to occur. Wherein the expression vector further includes a selectable marker
30 gene, the transformed or transfected cell line may be incubated on a media which at least

comprises a compound against which the selectable marker gene confers resistance, thereby facilitating the selection of cells which contain the expression vector and express the selectable marker gene at least.

- 5 The recombinant polypeptide thus produced may be partially-purified or purified to substantial homogeneity from the cell in which it is produced, using the method described by the present inventors for the purification of platelet heparanase (Example 1) or a modification thereof.
- 10 Alternatively, wherein the recombinant polypeptide is expressed as a fusion polypeptide, it is also possible to purify the fusion polypeptide based upon its properties (eg size, solubility, charge etc). Alternatively, the fusion polypeptide may be purified based upon the properties of the non-endoglucuronidase moiety of said fusion polypeptide, for example substrate affinity. Once purified, the fusion polypeptide may be cleaved to release the intact
- 15 endoglucuronidase polypeptide of the invention.

The isolated or purified recombinant endoglucuronidase polypeptide, in particular recombinant heparanase, is useful for any application wherein it is desirable to inhibit neovascularisation and its associated processes in the regulation of tissue development,

20 inflammation, wound healing and /or tumour metastasis.

Additionally, the isolated or purified recombinant endoglucuronidase polypeptide, in particular recombinant heparanase, may be used to assist in the determination of the structure and/or sequence of sulphated molecules, particularly those sulphated molecules which at least

25 comprise sulphated proteoglycans, sulphated oligosaccharides or heparan sulphate residues or side-chains, amongst others. By taking advantage of the functional nature of the recombinant polypeptide, a wide range of sulphated molecules may be subjected to digestion in the presence of the recombinant polypeptide of the invention for a time and under conditions sufficient to cleave the sulphated oligosaccharide moiety therefrom which may, if

30 necessary, be subjected to ultrastructure determination using mass spectrometry, infrared

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spectroscopy, nuclear magnetic resonance (NMR) spectroscopy or ultraviolet spectroscopy, amongst other methods known to those skilled in the art.

Additionally, recombinant endoglucuronidase polypeptide, in particular recombinant
5 heparanase, may be used in the preparation of immunologically interactive molecules, such as antibodies or functional derivatives thereof including Fabs or SCABS (single-chain antibodies), antibodies conjugated to an enzyme, radioactive or fluorescent tag. The present invention extends to recombinant and synthetic antibodies and to antibody hybrids. A "synthetic antibody" is considered herein to include fragments and hybrids of antibodies.

10

Both polyclonal and monoclonal antibodies are obtainable by immunisation with an appropriate recombinant polypeptide or an epitope thereof or a peptide fragment thereof, using procedures well-known to those skilled in the art.

15

Accordingly, the present invention clearly extends to immunologically-interactive molecules which are capable of binding to a mammalian recombinant endoglucuronidase or heparanase polypeptide.

20 Most preferably, the immunologically interactive molecule is an antibody molecule. The antibody molecule may be monoclonal or polyclonal and may be used for developing enzyme-linked immunosorbent assays (ELISA) or other immunoassays for the rapid diagnosis of elevated heparanase expression in human or animal cells and tissues to assist in the diagnosis of conditions associated therewith, such as angiogenesis, angioplasty-induced restenosis,
25 atherosclerotic plaque formation and inflammation, amongst others. The invention described herein extends to all such uses of immunointeractive molecules and diagnostic assays which require said immunoassays for their performance.

A wide range of immunoassay techniques may be such as those described in US Patent Nos.
30 4,016,043, 4,424,279 and 4,018,653. By way of example only, an antibody raised against

recombinant platelet heparanase is immobilised onto a solid substrate and a biological sample from an animal to be tested for the presence of elevated heparanase expression, for example serum or isolated platelets, is brought into contact with the bound molecule. After a suitable period of incubation, for a period of time sufficient to allow formation of an antibody-antigen
5 complex, a second antibody labelled with a reporter molecule capable of producing a detectable signal is then added and incubated, allowing sufficient time for the formation of a tertiary complex of antibody-antigen-labelled antibody. Any unreacted material is washed away, and the presence of the tertiary complex is determined by observation of a signal produced by the reporter molecule. The results may either be qualitative, by simple
10 observation of the visible signal or may be quantitated by comparison with a control sample containing known amounts of heparanase. Variations of this assay include a simultaneous assay, in which both sample and labelled antibody are added simultaneously to the bound antibody, or a reverse assay in which the labelled antibody and sample to be tested are first combined, incubated and then added simultaneously to the bound antibody. These techniques
15 are well known to those skilled in the art, and the possibility of minor variations will be readily apparent.

The solid substrate is typically glass or a polymer, the most commonly used polymers being cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene. The
20 solid supports may be in the form of tubes, beads, discs or microplates, or any other surface suitable for conducting an immunoassay. The binding processes are well-known in the art and generally consist of cross-linking covalently binding or physically adsorbing the molecule to the insoluble carrier.

25 By "reporter molecule", as used in the present specification, is meant a molecule which, by its chemical nature, produces an analytically identifiable signal which allows the detection of antigen-bound antibody. Detection may be either qualitative or quantitative. The most commonly used reporter molecule in this type of assay are either enzymes, fluorophores or radionuclide containing molecules (i.e. radioisotopes). In the case of an enzyme
30 immunoassay, an enzyme is conjugated to the second antibody, generally by means of

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glutaraldehyde or periodate. As will be readily recognised, however, a wide variety of different conjugation techniques exist which are readily available to one skilled in the art. Commonly used enzymes include horseradish peroxidase, glucose oxidase, β -galactosidase and alkaline phosphatase, amongst others. The substrates to be used with the specific
5 enzymes are generally chosen for the production, upon hydrolysis by the corresponding enzyme, of a detectable colour change. It is also possible to employ fluorogenic substrates, which yield a fluorescent product.

Alternatively, fluorescent compounds, such as fluorescein and rhodamine, may be chemically
10 coupled to antibodies without altering their binding capacity. When activated by illumination with light of a particular wavelength, the fluorochrome-labelled antibody adsorbs the light energy, inducing a state of excitability in the molecule, followed by emission of the light at a characteristic colour visually detectable with a light microscope. As in the EIA, the fluorescent labelled antibody is allowed to bind to the first antibody-hapten complex. After
15 washing off the unbound reagent, the remaining complex is then exposed to the light of the appropriate wavelength, the fluorescence observed indicates the presence of the hapten of interest. Immunofluorescence and EIA techniques are both very well established in the art and are particularly preferred for the present method. However, other reporter molecules, such as radioisotope, chemiluminescent or bioluminescent molecules, may also be employed.
20 It will be readily apparent to the skilled technician how to vary the procedure to suit the required purpose.

The immunologically-interactive molecule is also useful in purifying the recombinant heparanase of the present invention. Methods for the affinity purification of proteins using
25 antibodies are well-known to those skilled in the art.

In a further embodiment, the isolated nucleic acid molecule of the invention is placed in the antisense orientation relative to the promoter sequence to which it is operably connected such that when said nucleic acid molecule is expressed, an antisense molecule or ribozyme
30 molecule is transcribed.

In the context of the present invention, an antisense molecule is an RNA molecule which is transcribed from the complementary strand of a nuclear gene to that which is normally transcribed to produce a "sense" mRNA molecule capable of being translated into a polypeptide. The antisense molecule is therefore complementary to the sense mRNA, or a part thereof. Although not limiting the mode of action of the antisense molecules of the present invention to any specific mechanism, the antisense RNA molecule possesses the capacity to form a double-stranded mRNA by base pairing with the sense mRNA, which may prevent translation of the sense mRNA and subsequent synthesis of a polypeptide gene product.

Ribozymes are synthetic RNA molecules which comprise a hybridising region complementary to two regions, each of at least 5 contiguous nucleotide bases in the target sense mRNA. In addition, ribozymes possess highly specific endoribonuclease activity, which autocatalytically cleaves the target sense mRNA. A complete description of the function of ribozymes is presented by Haseloff and Gerlach (1988) and contained in International Patent Application No. WO89/05852. The present invention extends to ribozymes which target a sense mRNA encoding a mammalian endoglucuronidase polypeptide described herein, in particular human heparanase, thereby hybridising to said sense mRNA and cleaving it, such that it is no longer capable of being translated to synthesise a functional polypeptide product.

According to this embodiment, the present invention provides a ribozyme or antisense molecule comprising a sequence of contiguous nucleotide bases which are able to form a hydrogen-bonded complex with a part of the endoglucuronidase or heparanase mRNA at least about 10 to 20 contiguous nucleotides derived from any one of <400> 12 or <400> 14 or <400> 16 or <400> 18 or a complementary sequence thereto, preferably at least about 20-50 contiguous nucleotides derived from any one of <400> 12 or <400> 14 or <400> 16 or <400> 18 or a complementary sequence thereto, or more preferably at least about 50-500 contiguous nucleotides derived from any one of <400> 12 or <400> 14 or <400> 16 or <400> 18 or a complementary sequence thereto, or still more preferably to the full-length

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or substantially full-length endoglucuronidase or heparanase mRNA sequence.

It is understood in the art that certain modifications, including nucleotide substitutions amongst others, may be made to the antisense and/or ribozyme molecules of the present invention, without destroying the efficacy of said molecules in inhibiting the expression of an endoglucuronidase gene, in particular a human heparanase gene. It is therefore within the scope of the present invention to include any nucleotide sequence variants, homologues, analogues, or fragments of the said gene encoding same, the only requirement being that said nucleotide sequence variant, when transcribed, produces an antisense and/or ribozyme molecule which is capable of hybridising to the said sense mRNA molecule.

The ribozyme and antisense molecules of the invention are particularly useful in the prophylactic and therapeutic treatment of conditions associated with the elevated expression of heparanase in human or animal cells, such as metastasis, angiogenesis, angioplasty-induced restenosis, atherosclerotic plaque formation and inflammation, amongst others. According to this embodiment, the subject antisense or ribozyme molecule or a genetic construct expressing same may be administered to a human or animal subject for a time and under conditions sufficient to reduce or prevent the expression of the endogenous heparanase enzyme at an inflammation site, tumour site, in the extracellular matrix or endothelial surface, amongst others.

In the case of "naked" antisense or ribozyme molecules administered directly to the subject, those skilled in the art are aware that it may be necessary to include modified nucleotide residues, nucleotide analogues or other substituents to reduce or inhibit or prevent degradation of said molecules by cellular nuclease enzymes, thereby increasing their half-life following administration. Such modified nucleic acid molecules are well-known to those skilled in the art.

In the case of genetic constructs which express the subject antisense or ribozyme molecules

described herein, those skilled in the art will be aware that it will be important for the antisense or ribozyme molecule to be expressed following its administration to the subject, in order to achieve the advantageous effects of the invention in reducing heparanase expression.

5

Still yet another aspect of the invention contemplates a method of identifying a modulator of heparanase activity, said method comprising assaying recombinant heparanase activity in the presence of a potential modulator and comparing said activity to the activity of recombinant heparanase in the absence of said potential modulator.

10

As used herein, the term "modulator" shall be taken to refer to any chemical compound, molecule or macromolecule which is capable of altering the enzyme activity of an endoglucuronidase polypeptide, in particular a heparanase polypeptide, including both agonists and antagonists of said enzyme activity.

15

Preferably, the subject method further comprises the first step of expressing a functional recombinant endoglucuronidase polypeptide or heparanase polypeptide in a cell for a time and under conditions sufficient for said polypeptide to be produced in an assayable quantity.

20 The term "assayable quantity" refers to a level of expression of a recombinant polypeptide which is sufficient for the activity of said polypeptide to be determined by any standard enzyme assay procedure which is specific for the enzymic function of the recombinant polypeptide.

25 In a particularly preferred embodiment of the invention, the modulator is an antagonist molecule. According to this embodiment, the recombinant heparanase activity detected in the presence of said modulator is significantly less than that detected in the absence of said modulator, under substantially similar reaction conditions.

30 Preferred modulators of an endoglucuronidase or heparanase enzyme activity are capable of

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inhibiting or reducing said enzyme activity as measured *in vitro* or *in vivo* by at least about 20%, more preferably by at least about 50% and even more preferably by at least about 80%, compared to the enzyme activity which is detectable in the absence of said modulator.

- 5 In an alternative embodiment, the modulator of an endoglucuronidase or heparanase enzyme activity is capable of inhibiting or reducing said enzyme activity to a level sufficient to significantly reduce the level of neovascularisation and/or the proliferation of smooth muscle cells or alternatively, to reduce the level of degradation of endothelial cell surface HSPG and/or extracellular matrix HSPG by at least about 20%, more preferably by at least about
10 50% and even more preferably by at least about 80%.

A further aspect of the invention contemplates an inhibitor of a mammalian endoglucuronidase polypeptide enzyme activity, in particular a mammalian heparanase.

15

As used herein, the term "inhibitor" refers to any modulator of enzyme activity as hereinbefore defined or a nucleic acid molecule, such as a nucleic acid molecule which is capable of reducing the level of expression of a mammalian endoglucuronidase polypeptide, in particular a heparanase polypeptide in a cell, tissue or organ, wherein the reduced
20 expression leads to a reduction in the level of assayable endoglucuronidase or heparanase enzyme activity.

The inhibitor molecule of the present invention may be a non-cleavable substrate of a heparanase polypeptide or a negatively-charged molecule such as a sulphated oligosaccharide, sulphonate, phosphate or phosphonate, amongst others, or alternatively an antibody molecule
25 or catalytic antibody molecule capable of binding and inhibiting the activity of a heparanase polypeptide or alternatively, a nucleic acid inhibitor molecule such as a ribozyme, minizyme or antisense molecule, amongst others which is capable of inhibiting the expression of a heparanase polypeptide in a cell at the nucleic acid level, the only requirement being that said
30 inhibitor molecule is at least capable of reducing the activity of a heparanase polypeptide at

a wound site, tumour cell, extracellular matrix or endothelial surface, amongst others.

In a particularly preferred embodiment of the invention, the inhibitor molecule is a non-cleavable substrate or substrate analogue of a heparanase polypeptide, such as a sulphated
5 oligosaccharide, sulphonate or HSPG comprising same. More preferably, the inhibitor is one which is identified using the method described *supra* for the identification of modulators of endoglucuronidase enzyme activity.

The inhibitor molecules described herein is useful in a wide range of prophylactic and
10 therapeutic applications, by virtue of their ability to inhibit heparanase enzymes. The inhibitor molecules encompassed by the invention are particularly useful as inhibitors of metastasis, angiogenesis, wound healing, angioplasty-induced restenosis, arteriosclerosis, atherosclerosis, inflammation or other physiological or medical condition wherein heparanase activity is elevated.

15

The advantageous effects of the invention are achieved by the administration of a pharmaceutical composition which at least comprises one or more of the inhibitory molecules described herein as an active ingredient, to a human or animal subject by injection, oral
ingestion (e.g. in medicated food material) or topical administration.

20

The compositions may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art. Such methods include the step of bringing into association the active ingredient with the carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing into association
25 the active ingredient with liquid carriers or finely divided solid carriers or both, and then if necessary shaping the product.

Compositions of the present invention suitable for oral administration may be presented as discrete units such as capsules, sachets or tablets each containing a predetermined amount of
30 the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous or

non-aqueous liquid. The active ingredient may also be presented as a bolus, electuary or paste.

A tablet may be made by compression or moulding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the
5 active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder (e.g. inert diluent, preservative disintegrant (e.g. sodium starch glycolate, cross-linked polyvinyl pyrrolidone, cross-linked sodium carboxymethyl cellulose) surface-active or dispersing agent. Moulded tablets may be made by moulding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent.

10

Tablets or powders or granules may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile. Additionally, sweeteners or dietary formulae may be included to improve their palatability to
15 a specific animal subject. Optionally, such solid compositions be provided with an enteric coating, to provide release in parts of the gut other than the stomach.

The active compounds may also be administered in dispersions prepared in glycerol, liquid polyethylene glycols, and/or mixtures thereof and in oils. Under ordinary conditions of storage
20 and use, these preparations contain a preservative to prevent the growth of microorganisms.

Pharmaceutical forms suitable for parenteral administration include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases the form must be sterile and must be
25 fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The
30 proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the

maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars
5 or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as
10 required, followed by filter sterilisation. Generally, dispersions are prepared by incorporating the various sterilised active ingredient(s) into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the
15 active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

The carriers, excipients and/or diluents utilised in the pharmaceutical compositions of the present invention should be acceptable for human or veterinary applications. Such carriers,
20 excipients and/or diluents are well-known to those skilled in the art. Carriers and/or diluents suitable for veterinary use include any and all solvents, dispersion media, aqueous solutions, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the composition is contemplated. Supplementary active ingredients
25 can also be incorporated into the compositions.

The compositions of this invention may include other agents conventional in the art. For example, compositions suitable for oral administration may include such further agents as dietary formulae, binders, sweeteners, thickeners, flavouring agents disintegrating agents,
30 coating agents, preservatives, lubricants and/or time delay agents. Suitable sweeteners include

sucrose, lactose, glucose, aspartame or saccharine. Suitable disintegrating agents include corn starch, methylcellulose, polyvinylpyrrolidone, xanthan gum, bentonite, alginic acid or agar. Suitable flavouring agents include peppermint oil, oil of wintergreen, cherry, orange or raspberry flavouring. Suitable coating agents include polymers or copolymers of acrylic acid
5 and/or methacrylic acid and/or their esters, waxes, fatty alcohols, zein, shellac or gluten. Suitable preservatives include sodium benzoate, vitamin E, alpha-tocopherol, ascorbic acid, methyl paraben, propyl paraben or sodium bisulphite. Suitable time delay agents include glyceryl monostearate or glyceryl distearate.

- 10 The present invention is further described with reference to the following non-limiting Examples.

EXAMPLE 1

Purification and characterisation of mammalian heparanase

15

Human platelet heparanase was purified according to the method of Freeman and Parish International Patent Application No PCT/AU97/00453]. Evidence of purity, as shown by SDS-PAGE, is depicted in Figure 1. All samples were reduced with dithiothreitol prior to electrophoresis.

20

Purified human platelet heparanase had a Mr of 50 kDa as determined by SDS-PAGE analysis (Figure 1) and by gel filtration. N-deglycosylation of the enzyme with recombinant N-glycosidase F obtained from Boehringer Mannheim (Sydney, Australia) resulted in a reduction in Mr to 40 kDa (Figure 1). This is consistent with a Mr of 42 kDa as predicted
25 from the cDNA sequence for the de-glycosylated mature enzyme which encoded for 6 putative N-glycosylation sites (see Example 3). No further reduction in the apparent size of the N-deglycosylated material was observed following concurrent O-glycosidase and neuraminidase treatment (Figure 1). The purified membrane bound enzyme also had a native Mr and subunit Mr of 50 kDa as determined by gel filtration and SDS-PAGE analysis under
30 reducing conditions (Figure 1).

EXAMPLE 2

N-terminal and tryptic digest sequence determination

Using the method of Hellman *et al.* (1995), in situ trypsin digestion of the 50kDa band
5 obtained following SDS-PAGE analysis of purified human platelet heparanase resulted in the
isolation of 11 peptides which were amino acid sequenced using a Perkin Elmer Applied
Biosystems Procise 494 protein sequencer. The 50kDa band was excised, passively
transferred to PVDF nylon membrane, and the N-terminal sequence obtained by the method
of Messer *et al.* (1997).

10

The amino acid sequences of the trypsin digest-generated peptides and the N-terminal
sequence are shown in Table 3 (i.e. <400> 1-11 corresponding to peptides 1-11, respectively
of Table 3).

15

Comparison of the peptides and the N-terminal sequence with the amino acid sequence data
base demonstrated no highly significant or consistent homologies with any known proteins.
Peptides 2 and 3 were identical except peptide 2 was one residue greater in length. Peptides
1 and 8 were identical except peptide 1 was two residues longer. Peptides 5 and 7 were
20 minor sequences associated with peptides 4 and 6. The sequences were highly reliable for
all the peptides with only a few residues being questionable. An interesting feature of peptide
10 was evidence for polymorphism at residues 2 and 3. This is not surprising as the platelet
heparanase was prepared from pooled platelet preparations from many human donors.

25

EXAMPLE 3

Cloning of human heparanase cDNA

A cDNA clone designated as clone c1 (ATCC number 514661) was obtained from the
American Tissue Type Collection, Maryland, USA. This cDNA clone was identified in a
30 BLAST search of the EST database, for nucleotide sequences which might possible encode

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one or more of the amino acid sequences of human platelet heparanase obtained as described in the preceding example (<400> 1-11). The c1 clone was shown by the present inventors to comprise nucleotide sequences capable of encoding at least four human platelet heparanase peptide sequences or sequences closely related thereto, in particular those sequences set forth
5 in <400>1, 2, 9 and 10. These data strongly suggested that clone c1 encoded at least a part of the human platelet heparanase polypeptide.

Subsequent experiments by the inventors revealed that the c1 clone was approximately 1.1 kb in length, comprising nucleotides 774 to 1711 of <400>12, encoding the C-terminal end of
10 heparanase.

The c1 clone was fully sequenced and utilized to design primers for PCR amplification of the 5' end of the mRNA. A fragment designated c λ , approximately 800 bp in length, was amplified from a λ gt11 human placental cDNA library (ATCC number HL 1008). The c λ
15 fragment was sequenced and shown to contain an overlapping 3' sequence with the partial cDNA clone, in particular nucleotides 1 to 816 of <400> 12.

The c λ fragment was used to obtain two putative full length clones (designated c2 and c9), from the λ gt11 human placental cDNA library, by hybridisation screening. Clone c9 encoded
20 for the full length heparanase polypeptide, however it contained a 115 bp deletion from nucleotides 1144 to 1258 of <400> 12. Clone c2 comprised nucleotides 1 to 1481 of <400> 12 and was thus truncated within 169 bp from the stop codon.

The full length cDNA and amino acid sequence of the heparanase enzyme was deduced
25 (<400> 12 and <400>13). The heparanase open reading frame set forth in <400> 12 is 1629 nucleotides long and encodes for a 543 amino acid protein. The nucleotide sequence set forth in <400> 12 contains a putative polyadenylation signal at positions 1679 to 1684.

Eight of the eleven isolated tryptic digest peptides and the N-terminal sequence of the isolated
30 enzyme were detected in the amino acid sequence encoded by the assembled full-length cDNA

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sequence (i.e. <400>1-3, <400>6, <400>8-11). Seven of these eight tryptic peptides were essentially identical to the amino acid sequence encoded by the cDNA sequence (i.e. <400>1-3 and <400>8-11). Peptide 6 was found as incomplete sequences in the cDNA sequence while peptides 4, 5 and 7 were not found. Whether these peptide sequences are derived from a protein impurity in the heparanase preparation, or represent differently spliced variants of the heparanase remains to be seen.

The mature isolated enzyme appears to be a truncated form with the N-terminus located 158 amino acid residues downstream from the putative initiation codon, because whilst the open reading frame extends from nucleotides 46 to 1674 of <400>12, the mature protein is encoded by nucleotides 517 to 1674 of <400>12. The predicted cDNA size encoding for the mature isolated protein (assuming there had been no C-terminus processing) is 42.2 kDa which is consistent with an apparent size of 40 kDa obtained when the human platelet enzyme was N-deglycosylated (Figure 1).

15

The lysine acid residue at position 158 of the immature polypeptide set forth in <400>13 forms the N-terminus of the mature human heparanase polypeptide. Putative N-linked glycosylation sites exist at Asn162, Asn178, Asn200, Asn217, Asn238 and Asn459 in the immature full-length polypeptide.

20

EXAMPLE 4

Tissue distribution of human heparanase mRNA

25 The expression of human heparanase mRNA was analysed by Northern blot of various human tissues.

Northern analysis of multiple human tissue blots (Clontech, Palo Alto, CA) was performed by probing membranes with the full length human heparanase cDNA, labelled by random priming (Megaprime DNA labelling system, Amersham), using Expresshyb solution

30

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(Clontech) as specified by the manufacturers. Membranes were washed in 1xSSC for 40 minutes at room temperature followed by 0.1xSSC for 40 minutes at 60°C and exposed to X-ray film.

5 In non-immune tissues, a message of the expected size based on the isolated heparanase cDNA clone (~2kb), was detected in placenta but not in heart, brain, lung, liver, skeletal muscle, kidney or pancreas (Figure 3). A second message of 4.4kb was also detected in placenta but at a lower level than the 2kb message. The 4.4kb message was also detected weakly in all other tissues, and may represent an alternate splice variant or a product from
10 a related gene (see below). In immune tissues, both the 2kb and 4.4kb messages were detected in spleen, lymph node, thymus, peripheral blood leukocytes, bone marrow and fetal liver (Figure 4). The highest levels of mRNA were seen in PBL, with lower levels in spleen, lymph node, bone marrow and fetal liver, and only weak expression in the thymus. The expression levels of the 2kb and 4.4kb messages appeared similar in each of the immune
15 tissues, suggesting that both messages are derived from the same gene or from possibly from different genes that are coordinately regulated.

EXAMPLE 5

20 Southern blot analysis of the human heparanase gene

10µg of human genomic DNA was restricted with a range of restriction enzymes and separated on a 1% agarose gel then transferred to a Hybond-N nylon filter (Amersham, Arlington Heights, IL). The blot was probed with the full length human heparanase cDNA
25 labelled by random priming and hybridised in a 50% formamide, 6xSSC, 0.5% SDS, 5xDenhardt's solution and 100µg/ml salmon sperm DNA at 42°C. The membrane was washed in 1xSSC for 40 minutes at room temperature followed by 0.1xSSC for 40 minutes at 65°C and exposed to X-ray film.

30 Southern analysis of human genomic DNA from two individuals digested with a range of

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restriction enzymes and probed with the full length human heparanase cDNA, revealed a simple hybridising band pattern, consistent with the human heparanase gene being a single copy gene (Figure 5). Thus it is likely that the 4.4kb message observed by Northern analysis is a splice variant rather than a product from a related gene.

5

EXAMPLE 6

Cloning of Mouse and Rat Heparanase cDNAs

(1) Isolation of RNA and first strand cDNA synthesis

10 Total cellular RNA was prepared by homogenising 100mg of tissue or 10^7 cells in 1ml of Trizol reagent (Gibco-BRL), upon which aqueous fraction was recovered and RNA precipitated using isopropanol. First strand cDNA was produced from $5\mu\text{g}$ of total RNA by priming with an oligosaccharide dT primer (dT-Not, Table 2) using a First Strand cDNA synthesis system (Pharmacia Biotech) according to the manufacturers instructions.

15

(2) Polymerase chain reaction

Reactions were performed on 10ng of first strand cDNAs in the presence of 100ng of each oligonucleotide primer, 1.25 mM dNTPs, 50mM KCl, 10mM Tris-Cl pH 8.3 and 1.5mM MgCl_2 using 1 unit of Taq DNA polymerase (Bresatec) for 40 amplification cycles.

20

(3) Nucleotide sequencing

PCR products or cDNA clones were sequenced by automated sequencing using an Applied Biosystems 377 sequencer.

25 *(4) Cloning of cDNAs*

PCR products were subcloned directly into the T-tailed vector pCR2.1 (Invitrogen) as described by the manufacturer.

(5) Identification of mouse heparanase using bioinformatics and cDNA cloning by PCR

30 Mouse heparanase ESTs were identified by screening the dbest (public EST, GenBank)

database with the human heparanase nucleic acid sequence using BLASTN (Table 4). The EST nucleotide sequences were retrieved using ENTREZ (NCBI) and contiguous sequences assembled from overlapping ESTs. The compiled EST sequences covered the 3' end of the mouse heparanase cDNA and corresponded to nucleotides 1004 to the polyadenylated tail of
5 the human heparanase mRNA.

The nucleotide sequence of the mouse heparanase cDNA was extended by 513 bases towards the 5' end. This was achieved by performing PCR using the oligonucleotides BamHepN (corresponding to nucleotides 517-534 of the human heparanase cDNA) and mhep3
10 (corresponding to nucleotides 1234 to 1250 of the mouse heparanase cDNA (Table 5) on first strand cDNA made from total RNA isolated from activated 129 mouse spleen T cells.

The mouse heparanase cDNA fragment was sequenced directly and determined to be 1368 nucleotides in length (with nucleotides 1 to 513 being identical to that compiled from the
15 ESTs) and to encode 386 amino acids of the C-terminal portion of the molecule (corresponding to amino acids 158-543 of human heparanase which comprises the predicted mature protein).

The nucleotide sequence and derived amino acid sequence of the murine heparanase cDNA
20 are set forth in <400>16 and <400>17, respectively.

The predicted amino acid sequence contains 3 putative N-linked glycosylation sites at Asn37, Asn154 and Asn296 and a putative transmembrane region encompassed by residues 352-371. Alignment of the mouse and human heparanase amino acids sequences using PILEUP
25 (NCBI)) indicated 80.8% identity (Figure 5).

A clone of the mouse heparanase cDNA fragment (designated muhep-pCR2.1/1) was generated by subcloning the PCR fragment into the vector pCR2.1. The nucleotide sequence of this clone was identical to the sequence determined from the direct sequencing of the PCR
30 product.

(6) *Cloning of a rat heparanase cDNA clone by PCR*

A rat heparanase cDNA fragment was generated by performing 3' Rapid Amplification of cDNA Ends (RACE)-PCR using the BamHepN oligonucleotide and a poly-dT primer (dT-Not) (Table 5) on first strand DNA derived from the rat MAT tumour cell line.

5

The nucleotide sequence and derived amino acid sequence of the rat heparanase cDNA are set forth in <400>18 and <400>19, respectively. The rat heparanase cDNA was sequenced directly and determined to be 1168 nucleotides in length and encode 386 amino acids of the C-terminal portion of the molecule (corresponding to amino acids 158-543 of
10 human heparanase which comprises the mature protein).

The predicted amino acid sequence contains 2 putative N-linked glycosylation sites at Asn37 and Asn296 and, like the human and mouse heparanases contains a putative transmembrane region encompassed by residues 352-371. Alignment of the rat heparanase amino acid
15 sequence with that of the human and mouse reveals 79.7% and 93.7% identity respectively (Figure 6).

A clone of the rat heparanase cDNA fragment (designated rahep-pCR2.1/1) was generated by subcloning the PCR fragment into the vector pCR2.1. The nucleotide sequence of this
20 clone was identical to the sequence determined from the direct sequencing of the PCR product.

EXAMPLE 7

Baculovirus Expression of Mammalian Heparanase

25

(1) *Rat and Mouse Heparanases*

Both the rat and mouse heparanases (N-terminal coding sequences) were excised from their respective cloning vectors (rahep-pCR2.1/1 and muhep-pCR2.1/1) using the restriction enzymes *EcoRI* (mouse clone) and *BamHI/EcoRI* (rat clone). The excised fragments were
30 cloned into the plasmid pFastBac (Gibco BRL) in front of the polyhedron promotor and

transferred into the bacterial strain DH10Bac. Bacmid DNA (pFastBac integrated into the DH10Bac genome) was prepared and used to transfect Sf9 (*Spodoptera frugiperda*) insect cells. After 72 hours incubation the supernatant and the cells were harvested and used to test for enzyme activity.

5

Activity observed from the transfected cells versus untransfected cells for 2-3 separate samples is provided in Table 6.

Marginal heparanase activity was observed in 1/3 of the rat and mouse clones expressing the
10 N-terminal truncated sequence.

(2) Human N-terminal and Full length Heparanases

The two human constructs were excised from their respective T-tailed cloning vectors (NH2-
15 pCR2.1 and Full-pCR2.1) using the restriction enzyme *EcoRI*. The excised fragments were cloned into the plasmid pFastBac (Gibco BRL) in front of the polyhedron promotor and transferred into the bacterial strain DH10Bac. Bacmid DNA (pFastBac integrated into the DH10Bac genome) was prepared and used to transfect Sf9 (*Spodoptera frugiperda*) insect cells. After 72 hours incubation the supernatant and the cells were harvested and used to test
20 for enzyme activity.

Activity observed from the transfected cells versus untransfected cells for 2-5 separate samples is provided in Table 7.

25 Clean heparanase activity was observed in 3/5 clones containing the full length human heparanase sequence. Marginal heparanase activity was detected in 2/5 clones containing the N-terminal truncated sequence. Collectively, the baculovirus expression data suggests that the full length heparanase sequence is required to obtain best expression of active haparanase.

30

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EXAMPLE 8

Expression of Mammalian Heparanase in COS-7 Cells

(1) *Rat and Mouse Heparanases*

Both the rat and mouse heparanase cDNAs, which encode only the mature form of the heparanase protein (i.e. sequences homologous to the mature protein-encoding region of the human heparanase gene), were excised from their respective cloning vectors (rahep-pCR2.1/1 and muhep-pCR2.1/1) using the restriction enzymes *EcoRI* (mouse clone) and *BamHI/EcoRI* (rat clone). The excised fragments were cloned into the plasmid pcDNA3 (Invitrogen) in front of the cytomegalovirus (CMV) promoter. Plasmid DNA was prepared from *E. coli* DH5 α then used to transfect COS-7 mammalian cells using the following method.

COS-7 cells (30-50% confluent per 75cm² flask) were transiently transfected with the heparanase expression constructs or pcDNA3 vector alone, by the DEAE-dextran method as described. Cells were incubated with a transfection mixture (1ml/5cm² dish) consisting of 5-10 μ g/ml DNA, 0.4 mg/ml DEAE-dextran (Pharmacia) and 1 mM chloroquine (Sigma) in Dulbecco's Modified Eagles Medium (DME) (Flow Laboratories) containing 10% (v:v) Nuserum (Flow Laboratories) for 4 hr. The transfection mixture was then removed, cells treated for 2 min with 10% dimethylsulphoxide (v:v) in phosphate-buffered saline (PBS, 7.6mM Na₂HPO₄/3.25mM NaH₂PO₄/145mN NaCl), pH 7.4; washed and returned to fully supplemented culture medium for 48-72 hr before use in assays. COS-7 cells were maintained in DME supplemented with 10% heat-inactivated fetal calf serum, 100U/ml penicillin, 100mg/ml streptomycin, 2mM glutamine (Commonwealth Serum Laboratories) and 0.05mM 2-mercaptoethanol (2ME) (Koch-Light Ltd.). After 72 hours incubation the supernatant and the cells were harvested and used to test for enzyme activity.

25

The heparanase activity observed from the transfected cells versus mock transfected cells is shown in Table 8. These data indicate that no significant heparanase activity was apparent in transfected cells expressing the mouse or rat heparanase protein.

30

(2) *Human N-Terminal and Full Length Heparanases*

The two human constructs were excised from their respective T-tailed cloning vectors (NH2-pCR2.1, containing a human heparanase cDNA encoding amino acids 158-543 of <400> 13; 5 and Full-pCR2.1, containing a human heparanase cDNA encoding amino acids 1-543 of <400> 13 using the restriction enzyme *EcoRI*. The excised fragments were cloned into the plasmid pcDNA3 (Invitrogen) in front of the cytomegalovirus (CMV) promoter. Plasmid DNA was prepared from *E. coli* DH5 α then used to transfect COS-7 mammalian cells using the above method. After 72 hours incubation the supernatant and the cells were harvested and 10 used to test for enzyme activity.

The activity observed from the transfected cells versus untransfected cells is shown in Table 9. These data indicate that significant heparanase activity was present in COS-7 cells expressing the full length human heparanase sequence (ten times the background level), 15 however little or no activity was observed cells expressing only the mature processed form of the protein, as was observed for the rat and mouse proteins in COS-7 cells. Without being bound by any theory or mode of action, these data suggest an important functional role for amino acids 1 to 157 of human heparanases and probably the corresponding region of the rat and murine heparanases, in conferring correct expression and/or transport and/or processing 20 of the recombinant protein in mammalian cells.

EXAMPLE 9

25 Based on the comparative amino acid sequence data presented in Figure 6, a number of sequence differences were identified which could be used to prepare peptides for the raising of heparanase-specific antibodies. By way of exemplification only, a 15 amino acid peptide was synthesised that contained sequence differences between the human and mouse/rat heparanase sequences and contained a C-terminal cysteine residue which facilitated coupling 30 of the peptide to a protein carrier prior to immunizing rabbits. The amino acid sequence of

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the peptide, which spans residues 423 to 437 of the full length human heparanase sequence, is shown below:

VQGSKRRLRVYLHC (<400>23)

5

The 15 amino acid peptide was coupled to key hole limpet haemocyanin (KLH) via its C-terminal cysteine residue using Imject maleimide activated KLH (Pierce, Rockford, IL) according to the manufacturers instructions. The KLH-peptide conjugate dissolved in PBS (0.2mg/ml) was emulsified in Freund's Complete Adjuvant (FCA) at a 1:1 ratio of conjugate solution to FCA. Rabbits were immunized subcut in four sites with 0.2mg of KLH-peptide and the immunization repeated twice at 4 weekly intervals but using Freund's Incomplete Adjuvant rather than FCA, with rabbits being bled 2 weeks after the final immunization and the serum collected.

15 An ELISA assay was developed for assaying for anti-human heparanase antibodies. The assay involved immobilising human platelet heparanase (5µg/ml in PBS, 15 hr, 4°C), purified from human platelets as previously described, in 96 well plastic microplates (25 µl/well). Non-specific binding sites were then blocked by the addition of 200µl/well of PBS containing 1% (w/v) bovine serum albumin (BSA) for 2 hr at 4°C. Following three washes with 200µl/well of PBS/0.05% Tween 20 (PBST), 50µl/well of serial dilutions of the antisera in PBS/1% BSA were added and incubated for 2 hr at 4°C. Following three washes with PBST, 50µL/well of horse radish peroxidase (HRP) coupled sheep anti-rabbit Ig was added in PBS/1% BSA for 1 hr at 4°C, the plate again washed three times with PBST, and bound HRP measured by the addition of the colourimetric HRP substrate 2,2'-azino-bis (3-ethylbenthiazoline-6-sulfonic acid diammonium salt (ABTS), colour development being measured at 405nm on an ELISA plate reader after 30 minutes incubation at 37°C.

Figure 7 compares the ELISA results obtained with the anti-peptide antiserum with the reactivity of a polyclonal rabbit antibody raised against the purified human platelet heparanase. As shown in Figure 7, the anti-peptide antiserum exhibits considerable reactivity

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against the native enzyme, giving an endpoint titre of approx 1/640, compared with a titre of approx 1/10240 for the antiserum against the native enzyme. By comparison, serum obtained from rabbits prior to immunization with the peptide-KLH conjugate show negligible reactivity with the human heparanase.

TABLE 3
Sequences of Peptides Isolated from a Proteolytic Digest of
Human Platelet Heparanase

Peptide	Sequence	Comments
10 1. (10aa) ^a	1 5 10 LYGPDVGQPR	Reliable sequence
2. (12aa)	VFQVVESTTRPGK	Reliable sequence
15 3. (11aa)	VFQVVESTTRPG	Reliable sequence (same as peptide 2 less residue 12)
4. (7aa)	LPYQVQD	Mainly reliable sequence (? residue 4)
20 5. (7aa)	AGCQFIP	Minor sequence with peptide 4
6. (9aa)	LPYLFINLV	Reliable sequence
25 7. (8aa)	QNDPEDQL	Minor sequence with peptide 6
8. (8aa)	LYGPDVGQ	Reliable sequence (but incomplete). Same as peptide 1.
30 9. (12aa)	YLLRPLGPHEIN	Mainly reliable sequence (? residue 3)
10. (11aa)	V(Y/A)(L/A)HNTNTDNP	Mainly reliable sequence although reduced signal in later residues (residue 4 onwards). Polymorphism at residues 2 and 3.
35 11. (17aa) ^b	KKFKXSTYSRRSVDVLY	Amino-terminal sequence of enzyme

^a Number of amino acids (aa) in peptide

^b Amino-terminal sequence of complete heparanase enzyme prior to proteolytic digestion.

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TABLE 4
ESTs corresponding to mouse heparanase in Genbank

5		
	EST Accession No.	Tissue of Origin
	620141	spleen
10	1177651	mammary gland
	476953	embryo
	522550	skin
	1092868	diaphragm
15		

20

TABLE 5
Oligonucleotides used in cloning mouse and rat heparanase cDNAs

25		
	Oligonucleotide	Sequence
	BamHepN(<400> 20)	5'-AAAAAAGTTCAAGAACAGC-3'
30	mhep3 (<400> 21)	5'-CGAAGCTCTGGAACCTCG-3'
	dT-Not (<400> 22)	5'-AACTGGAAGAATTCGCGGCCGCAGGAAT-3'

- 60 -

TABLE 6
Recombinant Heparanase Expression in *Spodoptera frugiperda* cells
Transfected with Mammalian cDNA Clones

5	Heparanase cDNA	Heparanase Activity (pmol/hr/10⁶ cells)		
	Mouse	0.44	0.60	0.47
10	Rat	0.40	0.55	0.27
	Control	0.27	0.42	

15

20 **TABLE 7**
Recombinant Heparanase Activity in *Spodoptera frugiperda* Cells
Transfected with Full-length and Truncated Human Heparanase cDNA Clones

25	Gene Fragment	Heparanase Activity (pmol/hr/10⁶ cells)				
	Human (NH2 truncated)	0.46	0.39	0.50	0.57	0.43
	Human (Full-length)	0.22	0.97	1.12	0.76	0.39
30	Control	0.27	0.42			

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TABLE 8
Recombinant Heparanase Activity in COS-7 Cells Transfected with
Mouse and Rat Heparanase cDNA Clones

5

Heparanase cDNA	Heparanase Activity (pmol/hr/10⁶ cells)
<hr/>	
10 Mouse	30.3
Rat	25.0
Control	27.0

15

TABLE 9
Recombinant Heparanase Activity in COS-7 Cells Transfected with
Full-Length and Truncated Human Heparanase cDNA Clones

20

Gene Fragment	Heparanase Activity (pmol/hr/10⁶ cells)
<hr/>	
Human (NH2 truncated)	24.6
Human (Full-Length)	217.8
30 Control	27.0

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1713

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10 <212> PRT

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-145

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-135

-130

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-120

-115

-110

Leu His Leu Val Ser Pro Ser Phe Leu Ser Val Thr Ile Asp Ala Asn

-105

-100

-95

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-85

-80

Arg Thr Leu Ala Arg Gly Leu Ser Pro Ala Tyr Leu Arg Phe Gly Gly

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-75

-70

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Thr Lys Thr Asp Phe Leu Ile Phe Asp Pro Lys Lys Glu Ser Thr Phe

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-55

-50

35 Glu Glu Arg Ser Tyr Trp Gln Ser Gln Val Asn Gln Asp Ile Cys Lys

-45

-40

-35

-30

Tyr Gly Ser Ile Pro Pro Asp Val Glu Glu Lys Leu Arg Leu Glu Trp

-25

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-15

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- 74 -

Pro Tyr Gln Glu Gln Leu Leu Leu Arg Glu His Tyr Gln Lys Lys Phe
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Ala Asn Cys Ser Gly Leu Asp Leu Ile Phe Gly Leu Asn Ala Leu Leu
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10 Arg Thr Ala Asp Leu Gln Trp Asn Ser Ser Asn Ala Gln Leu Leu Leu
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Asp Tyr Cys Ser Ser Lys Gly Tyr Asn Ile Ser Trp Glu Leu Gly Asn
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15 Glu Pro Asn Ser Phe Leu Lys Lys Ala Asp Ile Phe Ile Asn Gly Ser
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Gln Leu Gly Glu Asp Phe Ile Gln Leu His Lys Leu Leu Arg Lys Ser
 20 85 90 95

Thr Phe Lys Asn Ala Lys Leu Tyr Gly Pro Asp Val Gly Gln Pro Arg
 100 105 110 115

25 Arg Lys Thr Ala Lys Met Leu Lys Ser Phe Leu Lys Ala Gly Gly Glu
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Val Ile Asp Ser Val Thr Trp His His Tyr Tyr Leu Asn Gly Arg Thr
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30 Ala Thr Arg Glu Asp Phe Leu Asn Pro Asp Val Leu Asp Ile Phe Ile
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Ser Ser Val Gln Lys Val Phe Gln Val Val Glu Ser Thr Arg Pro Gly
 35 165 170 175

Lys Lys Val Trp Leu Gly Glu Thr Ser Ser Ala Tyr Gly Gly Gly Ala
 180 185 190 195

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- 75 -

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Leu Gly Leu Ser Ala Arg Met Gly Ile Glu Val Val Met Arg Gln Val
5 215 220 225

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230 235 240

10 Leu Pro Asp Tyr Trp Leu Ser Leu Leu Phe Lys Lys Leu Val Gly Thr
245 250 255

Lys Val Leu Met Ala Ser Val Gln Gly Ser Lys Arg Arg Lys Leu Arg
260 265 270 275

15 Val Tyr Leu His Cys Thr Asn Thr Asp Asn Pro Arg Tyr Lys Glu Gly
280 285 290

Asp Leu Thr Leu Tyr Ala Ile Asn Leu His Asn Val Thr Lys Tyr Leu
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Arg Leu Pro Tyr Pro Phe Ser Asn Lys Gln Val Asp Lys Tyr Leu Leu
310 315 320

25 Arg Pro Leu Gly Pro His Gly Leu Leu Ser Lys Ser Val Gln Leu Asn
325 330 335

Gly Leu Thr Leu Lys Met Val Asp Asp Gln Thr Leu Pro Pro Leu Met
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30 Glu Lys Pro Leu Arg Pro Gly Ser Ser Leu Gly Leu Pro Ala Phe Ser
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<213> Homo sapiens

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<222> (52)..(1647)

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Leu Arg Ser Lys Pro Ala Leu Pro Pro Pro Leu Met Leu Leu Leu Leu
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Gly Pro Leu Gly Pro Leu Ser Pro Gly Ala Leu Pro Arg Pro Ala Gln
20          25          30

gca cag gac gtc gtg gac ctg gac ttc ttc acc cag gag ccg ctg cac 201
Ala Gln Asp Val Val Asp Leu Asp Phe Phe Thr Gln Glu Pro Leu His
35          40          45          50

25 ctg gtg agc ccc tcg ttc ctg tcc gtc acc att gac gcc aac ctg gcc 249
Leu Val Ser Pro Ser Phe Leu Ser Val Thr Ile Asp Ala Asn Leu Ala
55          60          65

acg gac ccg cgg ttc ctc atc ctc ctg ggt tct cca aag ctt cgt acc 297
30 Thr Asp Pro Arg Phe Leu Ile Leu Leu Gly Ser Pro Lys Leu Arg Thr
70          75          80

ttg gcc aga ggc ttg tct cct gcg tac ctg agg ttt ggt ggc acc aag 345
Leu Ala Arg Gly Leu Ser Pro Ala Tyr Leu Arg Phe Gly Gly Thr Lys
35          85          90          95

aca gac ttc cta att ttc gat ccc aag aag gaa tca acc ttt gaa gag 393
Thr Asp Phe Leu Ile Phe Asp Pro Lys Lys Glu Ser Thr Phe Glu Glu
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- 77 -

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 tcc atc cct cct gat gtg gag gag aag tta cgg ttg gaa tgg ccc tac 489
 Ser Ile Pro Pro Asp Val Glu Glu Lys Leu Arg Leu Glu Trp Pro Tyr
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 cag gag caa ttg cta ctc cga gaa cac tac cag aaa aag ttc aag aac 537
 Gln Glu Gln Leu Leu Leu Arg Glu His Tyr Gln Lys Lys Phe Lys Asn
 150 155 160

agc acc tac tca aga agc tct gta gat gtg cta tac act ttt gca aac 585
 15 Ser Thr Tyr Ser Arg Ser Ser Val Asp Val Leu Tyr Thr Phe Ala Asn
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 Cys Ser Gly Leu Asp Leu Ile Phe Gly Leu Asn Ala Leu Leu Arg Thr
 20 180 185 190

gca gat ttg cag tgg aac agt tct aat gct cag ttg ctc ctg gac tac 681
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gga gaa gat ttt att caa ttg cat aaa ctt cta aga aag tcc acc ttc 825
 35 Gly Glu Asp Phe Ile Gln Leu His Lys Leu Leu Arg Lys Ser Thr Phe
 245 250 255

aaa aat gca aaa ctc tat ggt cct gat gtt ggt cag cct cga aga aag 873
 Lys Asn Ala Lys Leu Tyr Gly Pro Asp Val Gly Gln Pro Arg Arg Lys
 40 260 265 270

- 78 -

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 Thr Ala Lys Met Leu Lys Ser Phe Leu Lys Ala Gly Gly Glu Val Ile
 275 280 285 290

5 gat tca gtt aca tgg cat cac tac tat ttg aat gga cgg act gct acc 969
 Asp Ser Val Thr Trp His His Tyr Tyr Leu Asn Gly Arg Thr Ala Thr
 295 300 305

agg gaa gat ttt cta aac cct gat gta ttg gac att ttt att tca tct 1017
 10 Arg Glu Asp Phe Leu Asn Pro Asp Val Leu Asp Ile Phe Ile Ser Ser
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 15 325 330 335

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 340 345 350

20 cta tcc gac acc ttt gca gct ggc ttt atg tgg ctg gat aaa ttg ggc 1161
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25 ctg tca gcc cga atg gga ata gaa gtg gtg atg agg caa gta ttc ttt 1209
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 375 380 385

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 Asp Tyr Trp Leu Ser Leu Leu Phe Lys Lys Leu Val Gly Thr Lys Val
 35 405 410 415

tta atg gca agc gtg caa ggt tca aag aga agg aag ctt cga gta tac 1353
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 435 440 445 450

5 act ctg tat gcc ata aac ctc cat aat gtc acc aag tac ttg cgg tta 1449
 Thr Leu Tyr Ala Ile Asn Leu His Asn Val Thr Lys Tyr Leu Arg Leu
 455 460 465

ccc tat cct ttt tct aac aag caa gtg gat aaa tac ctt cta aga cct 1497
 10 Pro Tyr Pro Phe Ser Asn Lys Gln Val Asp Lys Tyr Leu Leu Arg Pro
 470 475 480

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 15 485 490 495

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20 cct ctc cgg cca gga agt tca ctg ggt tgc cag ctt tct cat ata gtt 1641
 Pro Leu Arg Pro Gly Ser Ser Leu Gly Cys Gln Leu Ser His Ile Val
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 Phe Leu

gtcttgacac tgaaaaaaaa aaaaaa 1723

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 5 35 40 45

Leu His Leu Val Ser Pro Ser Phe Leu Ser Val Thr Ile Asp Ala Asn
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10 Leu Ala Thr Asp Pro Arg Phe Leu Ile Leu Leu Gly Ser Pro Lys Leu
 65 70 75 80

Arg Thr Leu Ala Arg Gly Leu Ser Pro Ala Tyr Leu Arg Phe Gly Gly
 85 90 95

15 Thr Lys Thr Asp Phe Leu Ile Phe Asp Pro Lys Lys Glu Ser Thr Phe
 100 105 110

Glu Glu Arg Ser Tyr Trp Gln Ser Gln Val Asn Gln Asp Ile Cys Lys
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Tyr Gly Ser Ile Pro Pro Asp Val Glu Glu Lys Leu Arg Leu Glu Trp
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25 Pro Tyr Gln Glu Gln Leu Leu Leu Arg Glu His Tyr Gln Lys Lys Phe
 145 150 155 160

Lys Asn Ser Thr Tyr Ser Arg Ser Ser Val Asp Val Leu Tyr Thr Phe
 165 170 175

30 Ala Asn Cys Ser Gly Leu Asp Leu Ile Phe Gly Leu Asn Ala Leu Leu
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Arg Thr Ala Asp Leu Gln Trp Asn Ser Ser Asn Ala Gln Leu Leu Leu
 35 195 200 205

Asp Tyr Cys Ser Ser Lys Gly Tyr Asn Ile Ser Trp Glu Leu Gly Asn
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- 81 -

Glu Pro Asn Ser Phe Leu Lys Lys Ala Asp Ile Phe Ile Asn Gly Ser
225 230 235 240

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Thr Phe Lys Asn Ala Lys Leu Tyr Gly Pro Asp Val Gly Gln Pro Arg
260 265 270

10 Arg Lys Thr Ala Lys Met Leu Lys Ser Phe Leu Lys Ala Gly Gly Glu
275 280 285

Val Ile Asp Ser Val Thr Trp His His Tyr Tyr Leu Asn Gly Arg Thr
290 295 300

15 Ala Thr Arg Glu Asp Phe Leu Asn Pro Asp Val Leu Asp Ile Phe Ile
305 310 315 320

Ser Ser Val Gln Lys Val Phe Gln Val Val Glu Ser Thr Arg Pro Gly
20 325 330 335

Lys Lys Val Trp Leu Gly Glu Thr Ser Ser Ala Tyr Gly Gly Gly Ala
340 345 350

25 Pro Leu Leu Ser Asp Thr Phe Ala Ala Gly Phe Met Trp Leu Asp Lys
355 360 365

Leu Gly Leu Ser Ala Arg Met Gly Ile Glu Val Val Met Arg Gln Val
370 375 380

30 Phe Phe Gly Ala Gly Asn Tyr His Leu Val Asp Glu Asn Phe Asp Pro
385 390 395 400

Leu Pro Asp Tyr Trp Leu Ser Leu Leu Phe Lys Lys Leu Val Gly Thr
35 405 410 415

Lys Val Leu Met Ala Ser Val Gln Gly Ser Lys Arg Arg Lys Leu Arg
420 425 430

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- 82 -

Val Tyr Leu His Cys Thr Asn Thr Asp Asn Pro Arg Tyr Lys Glu Gly
 435 440 445

5 Asp Leu Thr Leu Tyr Ala Ile Asn Leu His Asn Val Thr Lys Tyr Leu
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Arg Leu Pro Tyr Pro Phe Ser Asn Lys Gln Val Asp Lys Tyr Leu Leu
 465 470 475 480

10 Arg Pro Leu Gly Pro His Gly Leu Leu Ser Lys Ser Val Gln Leu Asn
 485 490 495

Gly Leu Thr Leu Lys Met Val Asp Asp Gln Thr Leu Pro Pro Leu Met
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30 <222> (1)..(1140)

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tcg ggg tta gac ctg atc ttt ggt cta aat gcg tta cta gga acc cca 96
 Ser Gly Leu Asp Leu Ile Phe Gly Leu Asn Ala Leu Leu Gly Thr Pro
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- 83 -

gac tta cgg tgg aac agc tcc aac gcc cag ctt ctc ctt gac tac tgc 144
 Asp Leu Arg Trp Asn Ser Ser Asn Ala Gln Leu Leu Leu Asp Tyr Cys
 35 40 45

5 tct tcc aag ggt tat aac atc tcc tgg gaa ctg ggc aat gag ccc aac 192
 Ser Ser Lys Gly Tyr Asn Ile Ser Trp Glu Leu Gly Asn Glu Pro Asn
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agt ttc tgg aag aaa gct cac att ctc atc gat ggg ttg cag tta gga 240
 10 Ser Phe Trp Lys Lys Ala His Ile Leu Ile Asp Gly Leu Gln Leu Gly
 65 70 75 80

gaa gac ttt gtg gag ttg cat aaa ctt cta caa agg tca gct ttc caa 288
 Glu Asp Phe Val Glu Leu His Lys Leu Leu Gln Arg Ser Ala Phe Gln
 15 85 90 95

aat gca aaa ctc tat ggt cct gac atc ggt cag cct cga ggg aag aca 336
 Asn Ala Lys Leu Tyr Gly Pro Asp Ile Gly Gln Pro Arg Gly Lys Thr
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20 gtt aaa ctg ctg agg agt ttc ctg aag gct ggc gga gaa gtg atc gac 384
 Val Lys Leu Leu Arg Ser Phe Leu Lys Ala Gly Gly Glu Val Ile Asp
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25 tct ctt aca tgg cat cac tat tac ttg aat gga cgc atc gct acc aaa 432
 Ser Leu Thr Trp His His Tyr Tyr Leu Asn Gly Arg Ile Ala Thr Lys
 130 135 140

gaa gat ttt ctg agc tct gat gtg ctg gac act ttt att ctc tct gtg 480
 30 Glu Asp Phe Leu Ser Ser Asp Val Leu Asp Thr Phe Ile Leu Ser Val
 145 150 155 160

caa aaa att ctg aag gtc act aaa gag atc aca cct ggc aag aag gtc 528
 Gln Lys Ile Leu Lys Val Thr Lys Glu Ile Thr Pro Gly Lys Lys Val
 35 165 170 175

tgg ttg gga gag acg agc tca gct tac ggt ggc ggt gca ccc ttg ctg 576
 Trp Leu Gly Glu Thr Ser Ser Ala Tyr Gly Gly Gly Ala Pro Leu Leu
 40 180 185 190

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tcc aac acc ttt gca gct ggc ttt atg tgg ctg gat aaa ttg ggc ctg 624
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 195 200 205

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tca gcc cag atg ggc ata gaa gtc gtg atg agg cag gtg ttc ttc gga 672
 Ser Ala Gln Met Gly Ile Glu Val Val Met Arg Gln Val Phe Phe Gly
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gca ggc aac tac cac tta gtg gat gaa aac ttt gag cct tta cct gat 720
 Ala Gly Asn Tyr His Leu Val Asp Glu Asn Phe Glu Pro Leu Pro Asp
 225 230 235 240

tac tgg ctc tct ctt ctg ttc aag aaa ctg gta ggt ccc agg gtg tta 768
 15 Tyr Trp Leu Ser Leu Leu Phe Lys Lys Leu Val Gly Pro Arg Val Leu
 245 250 255

ctg tca aga gtg aaa ggc cca gac agg agc aaa ctc cga gtg tat ctc 816
 Leu Ser Arg Val Lys Gly Pro Asp Arg Ser Lys Leu Arg Val Tyr Leu
 20 260 265 270

cac tgc act aac gtc tat cac cca cga tat cag gaa gga gat cta act 864
 His Cys Thr Asn Val Tyr His Pro Arg Tyr Gln Glu Gly Asp Leu Thr
 275 280 285

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 Pro Pro Leu Phe Arg Lys Pro Val Asp Thr Tyr Leu Leu Lys Pro Ser
 305 310 315 320

ggg ccg gat gga tta ctt tcc aaa tct gtc caa ctg aac ggt caa att 1008
 35 Gly Pro Asp Gly Leu Leu Ser Lys Ser Val Gln Leu Asn Gly Gln Ile
 325 330 335

ctg aag atg gtg gat gag cag acc ctg cca gct ttg aca gaa aaa cct 1056
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ctc ccc gca gga agt gca cta agc ctg cct gcc ttt tcc tat ggt ttt 1104
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Asp Leu Arg Trp Asn Ser Ser Asn Ala Gln Leu Leu Leu Asp Tyr Cys
 35 40 45
 Ser Ser Lys Gly Tyr Asn Ile Ser Trp Glu Leu Gly Asn Glu Pro Asn
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Ser Phe Trp Lys Lys Ala His Ile Leu Ile Asp Gly Leu Gln Leu Gly
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 Glu Asp Phe Val Glu Leu His Lys Leu Leu Gln Arg Ser Ala Phe Gln

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85 90 95

- 86 -

Asn Ala Lys Leu Tyr Gly Pro Asp Ile Gly Gln Pro Arg Gly Lys Thr
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5 Val Lys Leu Leu Arg Ser Phe Leu Lys Ala Gly Gly Glu Val Ile Asp
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Ser Leu Thr Trp His His Tyr Tyr Leu Asn Gly Arg Ile Ala Thr Lys
130 135 140

10 Glu Asp Phe Leu Ser Ser Asp Val Leu Asp Thr Phe Ile Leu Ser Val
145 150 155 160

Gln Lys Ile Leu Lys Val Thr Lys Glu Ile Thr Pro Gly Lys Lys Val
15 165 170 175

Trp Leu Gly Glu Thr Ser Ser Ala Tyr Gly Gly Gly Ala Pro Leu Leu
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20 Ser Asn Thr Phe Ala Ala Gly Phe Met Trp Leu Asp Lys Leu Gly Leu
195 200 205

Ser Ala Gln Met Gly Ile Glu Val Val Met Arg Gln Val Phe Phe Gly
210 215 220

25 Ala Gly Asn Tyr His Leu Val Asp Glu Asn Phe Glu Pro Leu Pro Asp
225 230 235 240

Tyr Trp Leu Ser Leu Leu Phe Lys Lys Leu Val Gly Pro Arg Val Leu
30 245 250 255

Leu Ser Arg Val Lys Gly Pro Asp Arg Ser Lys Leu Arg Val Tyr Leu
260 265 270

35 His Cys Thr Asn Val Tyr His Pro Arg Tyr Gln Glu Gly Asp Leu Thr
275 280 285

Leu Tyr Val Leu Asn Leu His Asn Val Thr Lys His Leu Lys Val Pro
40 290 295 300

- 87 -

Pro Pro Leu Phe Arg Lys Pro Val Asp Thr Tyr Leu Leu Lys Pro Ser
305 310 315 320

Gly Pro Asp Gly Leu Leu Ser Lys Ser Val Gln Leu Asn Gly Gln Ile
5 325 330 335

Leu Lys Met Val Asp Glu Gln Thr Leu Pro Ala Leu Thr Glu Lys Pro
340 345 350

10 Leu Pro Ala Gly Ser Ala Leu Ser Leu Pro Ala Phe Ser Tyr Gly Phe
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Phe Val Ile Arg Asp Ala Lys Ile Ala Ala Cys Ile
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tcg agg tta gac ctg atc ttt ggt cta aat gcg tta cta aga acc cca 96

Ser Arg Leu Asp Leu Ile Phe Gly Leu Asn Ala Leu Leu Arg Thr Pro

20 25 30

35

gac ttg cgg tgg aac agc tcc aac gcc cag ctt ctg ctc aac tac tgc 144

Asp Leu Arg Trp Asn Ser Ser Asn Ala Gln Leu Leu Leu Asn Tyr Cys

35 40 45

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- 88 -

tct tcc aag ggt tat aac atc tgc tgg gaa ctg ggc aac gag ccc aac 192
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5 agt ttc tgg aag aaa gct cac att tcc atc gat ggg ttg cag cta gga 240
 Ser Phe Trp Lys Lys Ala His Ile Ser Ile Asp Gly Leu Gln Leu Gly
 65 70 75 80

gaa gac ttt gtg gag ttg cat aaa ctt cta caa aag tca gct ttc caa 288
 10 Glu Asp Phe Val Glu Leu His Lys Leu Leu Gln Lys Ser Ala Phe Gln
 85 90 95

aac gca aaa ctc tat ggt cct gac att ggt cag cct cga ggg aag aca 336
 Asn Ala Lys Leu Tyr Gly Pro Asp Ile Gly Gln Pro Arg Gly Lys Thr
 15 100 105 110

gtt aag ctg ctg aga agc ttc ctg aag gct ggt gga gaa gtg att gac 384
 Val Lys Leu Leu Arg Ser Phe Leu Lys Ala Gly Gly Glu Val Ile Asp
 115 120 125

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 Ser Leu Thr Trp His His Tyr Tyr Leu Asn Gly Arg Val Ala Thr Lys
 130 135 140

25 gaa gat ttt ctg agc tct gat gtc ctg gac act ttt atc cta tct gtg 480
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 145 150 155 160

caa aaa att ctg aag gtg act aag gag atg aca cct ggc aag aag gtc 528
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 165 170 175

tgg ttg gga gag acg agc tct gcc tac ggc ggc gga gcg ccc ttg ctg 576
 35 Trp Leu Gly Glu Thr Ser Ser Ala Tyr Gly Gly Gly Ala Pro Leu Leu
 180 185 190

tcc gat acc ttt gca gct ggc ttt atg tgg ctg gat aaa ttg ggc ctg 624
 Ser Asp Thr Phe Ala Ala Gly Phe Met Trp Leu Asp Lys Leu Gly Leu
 40 195 200 205

- 89 -

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 210 215 220

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gca ggc aac tac cac tta gtg gac gaa aac ttc gag ccc ttg ccc gat 720
 Ala Gly Asn Tyr His Leu Val Asp Glu Asn Phe Glu Pro Leu Pro Asp
 225 230 235 240

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tac tgg ctc tct ctc ctg ttc aag aaa ctg gta ggt ccc aag gtg tta 768
 Tyr Trp Leu Ser Leu Leu Phe Lys Lys Leu Val Gly Pro Lys Val Leu
 245 250 255

atg tca aga gtg aaa ggc cca gac aga agc aaa ctc cga gtg tac ctc 816
 15 Met Ser Arg Val Lys Gly Pro Asp Arg Ser Lys Leu Arg Val Tyr Leu
 260 265 270

cac tgc acg aac gtc tat cac cca agg tat cgg gaa gga gat tta act 864
 His Cys Thr Asn Val Tyr His Pro Arg Tyr Arg Glu Gly Asp Leu Thr
 20 275 280 285

ctg tac gtc ctg aac ctc cat aat gtc acc aag cac ttg aag ctg ccg 912
 Leu Tyr Val Leu Asn Leu His Asn Val Thr Lys His Leu Lys Leu Pro
 290 295 300

25

cct ccg atg ttc agc aga ccg gtg gat aag tac ctg ctg aag cct ttc 960
 Pro Pro Met Phe Ser Arg Pro Val Asp Lys Tyr Leu Leu Lys Pro Phe
 305 310 315 320

30

ggt tct gac gga ctg ctt tcc aaa tcc gtc caa ctg aac ggt caa acc 1008
 Gly Ser Asp Gly Leu Leu Ser Lys Ser Val Gln Leu Asn Gly Gln Thr
 325 330 335

35

ctg aag atg gtc gat gag cag acc ctg cca gct cta aca gaa aaa cct 1056
 Leu Lys Met Val Asp Glu Gln Thr Leu Pro Ala Leu Thr Glu Lys Pro
 340 345 350

- 90 -

ctc ccc gca gga agc tca cta agc gtg ccc gcc ttt tcc tat ggg ttt 1104

Leu Pro Ala Gly Ser Ser Leu Ser Val Pro Ala Phe Ser Tyr Gly Phe

355

360

365

5 ttt gtc ata aga aat gcc aaa atc gca gct tgt ata tgaaaataaa 1150

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375

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15 <213> Rattus sp.

<400> 19

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Ser Arg Leu Asp Leu Ile Phe Gly Leu Asn Ala Leu Leu Arg Thr Pro

20

25

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Asp Leu Arg Trp Asn Ser Ser Asn Ala Gln Leu Leu Leu Asn Tyr Cys

25

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Ser Ser Lys Gly Tyr Asn Ile Cys Trp Glu Leu Gly Asn Glu Pro Asn

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30 Ser Phe Trp Lys Lys Ala His Ile Ser Ile Asp Gly Leu Gln Leu Gly

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80

Glu Asp Phe Val Glu Leu His Lys Leu Leu Gln Lys Ser Ala Phe Gln

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Asn Ala Lys Leu Tyr Gly Pro Asp Ile Gly Gln Pro Arg Gly Lys Thr

100

105

110

Val Lys Leu Leu Arg Ser Phe Leu Lys Ala Gly Gly Glu Val Ile Asp

40

115

120

125

- 91 -

Ser Leu Thr Trp His His Tyr Tyr Leu Asn Gly Arg Val Ala Thr Lys
 130 135 140

5 Glu Asp Phe Leu Ser Ser Asp Val Leu Asp Thr Phe Ile Leu Ser Val
 145 150 155 160

Gln Lys Ile Leu Lys Val Thr Lys Glu Met Thr Pro Gly Lys Lys Val
 165 170 175

10 Trp Leu Gly Glu Thr Ser Ser Ala Tyr Gly Gly Gly Ala Pro Leu Leu
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Ser Asp Thr Phe Ala Ala Gly Phe Met Trp Leu Asp Lys Leu Gly Leu
 15 195 200 205

Ser Ala Gln Leu Gly Ile Glu Val Val Met Arg Gln Val Phe Phe Gly
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20 Ala Gly Asn Tyr His Leu Val Asp Glu Asn Phe Glu Pro Leu Pro Asp
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Tyr Trp Leu Ser Leu Leu Phe Lys Lys Leu Val Gly Pro Lys Val Leu
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25 Met Ser Arg Val Lys Gly Pro Asp Arg Ser Lys Leu Arg Val Tyr Leu
 260 265 270

His Cys Thr Asn Val Tyr His Pro Arg Tyr Arg Glu Gly Asp Leu Thr
 30 275 280 285

Leu Tyr Val Leu Asn Leu His Asn Val Thr Lys His Leu Lys Leu Pro
 290 295 300

35 Pro Pro Met Phe Ser Arg Pro Val Asp Lys Tyr Leu Leu Lys Pro Phe
 305 310 315 320

Gly Ser Asp Gly Leu Leu Ser Lys Ser Val Gln Leu Asn Gly Gln Thr
 325 330 335

40

- 92 -

Leu Lys Met Val Asp Glu Gln Thr Leu Pro Ala Leu Thr Glu Lys Pro
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Leu Pro Ala Gly Ser Ser Leu Ser Val Pro Ala Phe Ser Tyr Gly Phe
5 355 360 365

Phe Val Ile Arg Asn Ala Lys Ile Ala Ala Cys Ile
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<210> 20
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<212> DNA
<213> Artificial Sequence

15

<220>
<223> Description of Artificial Sequence:OLIGONUCLEOTIDE

20 <400> 20

aaaaagtcca agaacagc

18

25 <210> 21

<211> 17

<212> DNA

<213> Artificial Sequence

30 <220>
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17

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<211> 28

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<213> Artificial Sequence

40 <220>

- 93 -

<223> Description of Artificial Sequence: OLIGONUCLEOTIDE

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28

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<210> 23

<211> 15

<212> PRT

10 <213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: PEPTIDE

15 <400> 23

Val Gln Gly Ser Lys Arg Arg Lys Leu Arg Val Tyr Leu His Cys

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WE CLAIM:

1. An isolated nucleic acid molecule that comprises a nucleotide sequence having at least 80% identity to any one of <400>12 or <400>14 or <400>16 or <400>18 or a complementary sequence thereto and which encodes a polypeptide having mammalian endoglucuronidase activity.
2. The isolated nucleic acid molecule of claim 1, wherein the endoglucuronidase activity is heparanase.
3. The isolated nucleic acid molecule of claim 2 comprising the nucleotide sequence set forth in <400>12 or a complementary nucleotide sequence thereto.
4. The isolated nucleic acid molecule of claim 2 comprising the nucleotide sequence set forth in <400>16 or a complementary nucleotide sequence thereto.
5. The isolated nucleic acid molecule of claim 2 comprising the nucleotide sequence set forth in <400>18 or a complementary nucleotide sequence thereto.
6. An isolated nucleic acid molecule that comprises a nucleotide sequence encoding a mammalian endoglucuronidase polypeptide comprising an amino acid sequence which is at least 80% identical to an amino acid sequence selected from the following:
 - (i) the amino acid sequence set forth in any one of <400>1 to <400>11 or <400>23;
 - (ii) the amino acid sequence set forth in <400>13;
 - (iii) the amino acid sequence set forth in <400>17;
 - (iv) the amino acid sequence set forth in <400>19;
 - (v) a homologue, analogue or derivative of any one of (i) to (iv) that is capable of removing the HS side chain from HSPG; and
 - (vi) a homologue, analogue or derivative of <400>15 that is capable of removing

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the HS side chain from HSPG.

7. The isolated nucleic acid molecule of claim 6, wherein the endoglucuronidase activity is heparanase.
8. The isolated nucleic acid molecule of claim 7 encoding the amino acid sequence set forth in <400> 13 or amino acid residues 158 to 543 of <400> 13.
9. The isolated nucleic acid molecule of claim 7 encoding the amino acid sequence set forth in <400> 17.
10. The isolated nucleic acid molecule of claim 7 encoding the amino acid sequence set forth in <400> 19.
11. A method of identifying a nucleic acid molecule encoding a mammalian endoglucuronidase polypeptide comprising at least the steps of:
 - (i) hybridising genomic DNA, mRNA or cDNA derived from a mammalian cell, tissue or organ with a hybridisation-effective amount of one or more probes or primer molecules comprising at least 10 contiguous nucleotides in length derived from any one of <400> 12 or <400> 14 or <400> 16 or <400> 18 for a time and under conditions sufficient for hybridisation to occur; and
 - (ii) detecting the hybridisation.
12. The method of claim 11 further comprising the step of isolating the hybridised nucleic acid molecule.
13. The method of claim 11 wherein the step of detecting the hybridisation comprises a polymerase chain reaction format.

14. The method of claim 11 wherein the step of detecting the hybridisation comprises a primer extension.
15. The method of claim 11 wherein the step of detecting the hybridisation comprises detecting a reporter molecule that is covalently bound to the probe or primer.
16. An expression vector comprising the isolated nucleic acid molecule of claim 1 operably linked to a promoter sequence.
17. The expression vector of claim 16 wherein the promoter is the polyhedron promoter or the CMV promoter.
18. An expression vector comprising the isolated nucleic acid molecule of claim 6 operably linked to a promoter sequence.
19. The expression vector of claim 18 wherein the promoter is the polyhedron promoter or the CMV promoter.
20. An expression vector comprising an isolated nucleic acid molecule that encodes a mammalian heparanase polypeptide having an amino acid sequence set forth in any one of <400> 13, <400> 15, <400> 17 or <400> 19 or a homologue, analogue or derivative thereof that is capable of removing the HS side chain from HSPG operably linked to a promoter sequence.
21. The expression vector of claim 20 wherein the promoter is the polyhedron promoter or the CMV promoter.
22. An isolated heparanase peptide comprising an amino acid sequence set forth in any one of <400> 1-11 or <400> 23.

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23. A recombinant or isolated polypeptide having endoglucuronidase activity and comprising an amino acid sequence that is at least 80% identical to any one of <400> 13, amino acids 158 to 543 of <400> 13, <400> 17 or <400> 19 or a homologue, analogue or derivative thereof that is capable of removing the HS side chain from HSPG.

24. The recombinant or isolated polypeptide of claim 23, wherein the endoglucuronidase activity comprises heparanase activity.

25. The isolated or recombinant polypeptide of claim 24 comprising the amino acid sequence set forth in <400> 13 or amino acids 158 to 543 of <400> 13.

26. The isolated or recombinant polypeptide of claim 24 comprising the amino acid sequence set forth in <400> 17.

27. The isolated or recombinant polypeptide of claim 24 comprising the amino acid sequence set forth in <400> 19.

28. The isolated or recombinant polypeptide of claim 24 comprising the mature protein region of the amino acid sequence set forth in <400> 13.

29. An antibody molecule which is capable of binding to an isolated or recombinant endoglucuronidase polypeptide that comprises an amino acid sequence that is at least 80% identical to any one of <400> 1-11, <400> 13, <400> 17 or <400> 19 or <400> 23.

30. A method of identifying a modulator of heparanase activity comprising assaying the activity of the recombinant endoglucuronidase enzyme of claim 23 in the presence of a potential modulator and comparing said activity to the activity of recombinant heparanase in the absence of said potential modulator.

31. The method of claim 30 wherein the modulator of heparanase activity is an inhibitor

of heparanase activity.

32. The method of claim 31 wherein the inhibitor of heparanase activity is a non-cleavable substrate or substrate analogue of heparanase.

33. The method of claim 31 wherein the inhibitor of heparanase activity is a sulfated oligosaccharide, a sulphonate or HSPG comprising same.

34. The method of claim 31 wherein the inhibitor of heparanase activity is an antibody molecule which is capable of binding to an isolated or recombinant endoglucuronidase polypeptide that comprises an amino acid sequence that is at least 80% identical to any one of <400> 1-11, <400> 13, <400> 17 or <400> 19 or <400> 23.

35. Use of a sulfated oligosaccharide, a sulphonate or HSPG comprising same to inhibit a heparanase polypeptide comprising an amino acid sequence that is at least 80% identical to any one of <400> 13, <400> 17 or <400> 19 or a homologue, analogue or derivative thereof that is capable of removing the HS side chain from HSPG.

36. A method of treatment of a physiological or medical condition in a human or animal subject wherein the heparanase activity in said subject is elevated, said method comprising administering an inhibitor of a heparanase polypeptide having an amino acid sequence that is at least 80% identical to any one of <400> 13, <400> 17 or <400> 19 for a time and under conditions sufficient for the heparanase activity in said subject to be reduced.

37. The method of claim 36 wherein the physiological or medical condition associated with elevated heparanase activity is selected from the list comprising metastasis, angiogenesis, wound healing, angioplasty-induced restenosis, arteriosclerosis, atherosclerosis and inflammation.

38. A method of enhancing wound healing in a human or animal subject, said method

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comprising administering to said subject a would healing enhancing amount of a recombinant or isolated heparanase polypeptide that comprises an amino acid sequence that is at least 80% identical to any one of <400> 13, <400> 17 or <400> 19 or a homologue, analogue or derivative thereof that is capable of releasing the HS side chain from HSPG or a pharmaceutical composition comprising said polypeptide, homologue, analogue or derivative.

39. The method of claim 38 wherein the would healing enhancement is associated with tissue development and tissue repair.

40. A method of diagnosing a physiological or medical condition associated with heparanase over-expression, said method comprising the steps of contacting the antibody of claim 29 with a biological sample derived from a human or animal subject suspected of suffering from said condition for a time and under conditions sufficient for an antigen:antibody complex to form and then detecting said complex formation.

41. The method of claim 40 wherein the physiological or medical condition is selected from the list comprising cancer, metastasis, angiogenesis, angioplasty-induced restenosis, atherosclerosis and inflammation.

42. The method of claim 40 wherein the biological sample is serum, placenta, peripheral blood leukocytes, spleen, lymph node, bone marrow or fetal liver or a derivative thereof.

43. A method of diagnosing, in a human or animal subject, a physiological or medical condition associated with heparanase over-expression, said method comprising the steps of:

- (i) contacting a mRNA-containing biological sample derived from a cell or tissue that expresses heparanase with a probe or primer that comprises a nucleotide sequence having at least 80% identity to at least 10 contiguous nucleotides of any one of <400> 12, <400> 14, <400> 16 or <400> 18 or a complementary nucleotide sequence thereto for a time and under conditions

- sufficient for hybridisation to occur; and
- (ii) detecting and/or quantifying the hybridisation.

44. The method of claim 43 wherein the physiological or medical condition is selected from the list comprising cancer, metastasis, angiogenesis, angioplasty-induced restenosis, atherosclerosis and inflammation.

45. The method of claim 44 wherein the biological sample comprises placenta, peripheral blood leukocytes, spleen, lymph node, bone marrow or fetal liver or a derivative thereof.

46. The method of claim 44 wherein the step of detecting and/or quantifying the hybridisation comprises comparing the hybridisation signal obtained for the subject to the hybridisation signal obtained for a healthy individual in a polymerase chain reaction format.

47. The method of claim 44 wherein the probe or primer includes a reporter molecule covalently bound to the nucleotide sequence and wherein the step of detecting and/or quantifying the hybridisation comprises comparing the amount of the reporter molecule that is bound to the biological sample derived from the subject to the amount of reporter molecule bound to an equivalent biological sample derived from a healthy individual.

48. A cell comprising the nucleic acid molecule according to any one of claims 1 to 10 or the expression vector according to any one of claims 16 to 21.

49. The cell according to claim 49 being an insect cell or a mammalian cell.

50. The cell according to claim 49 wherein the insect is *Spodoptera frugiperda* or the mammalian cell is a COS cell.

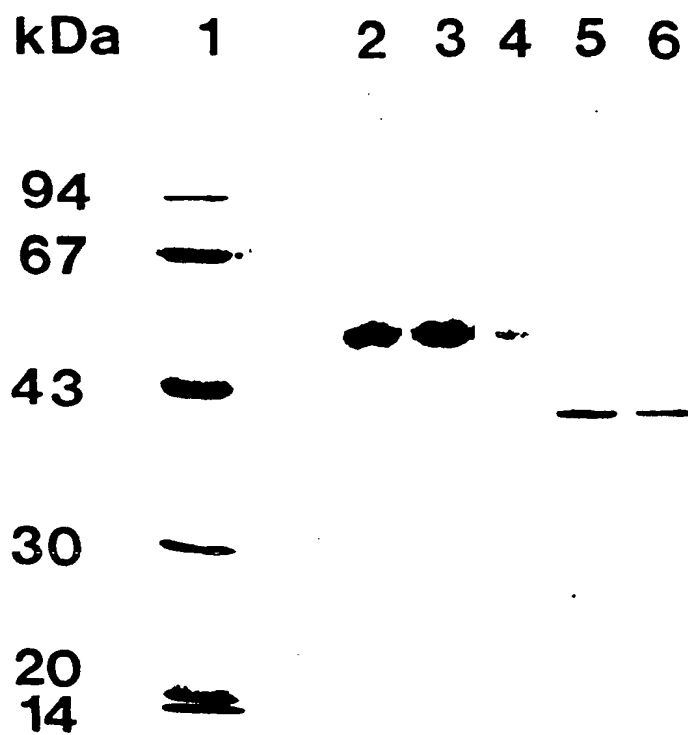


FIGURE 1

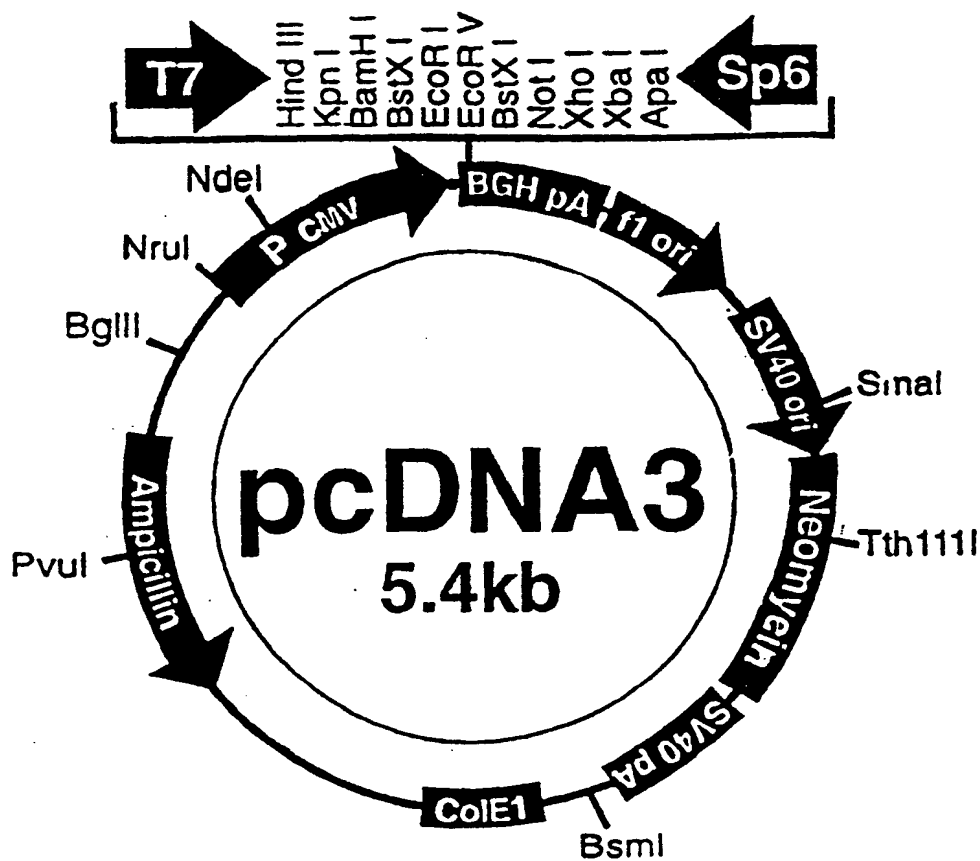
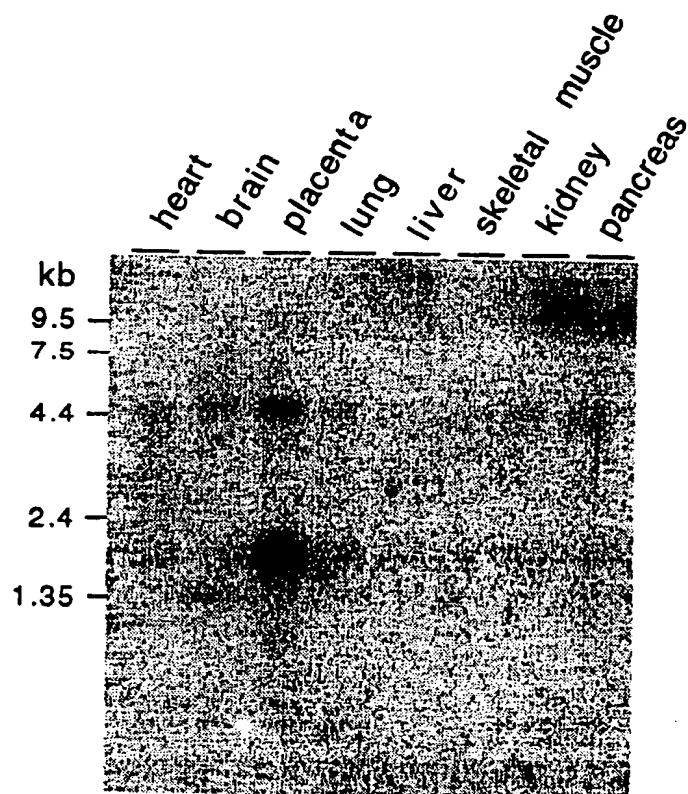
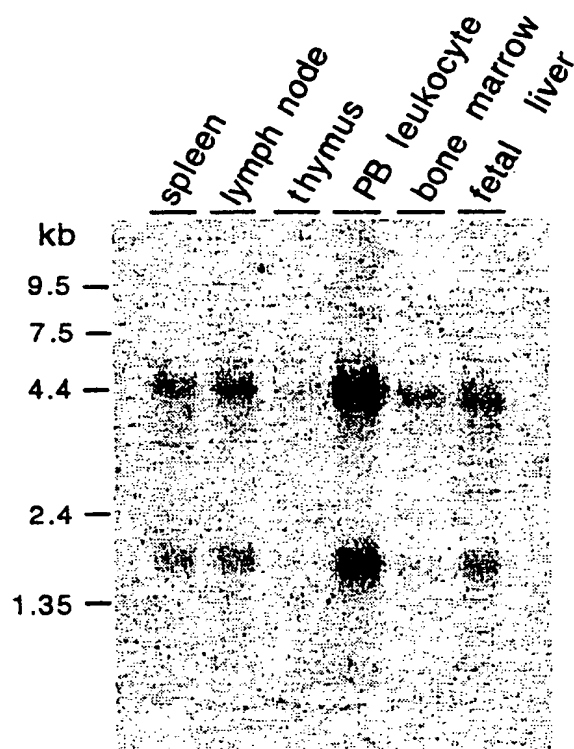


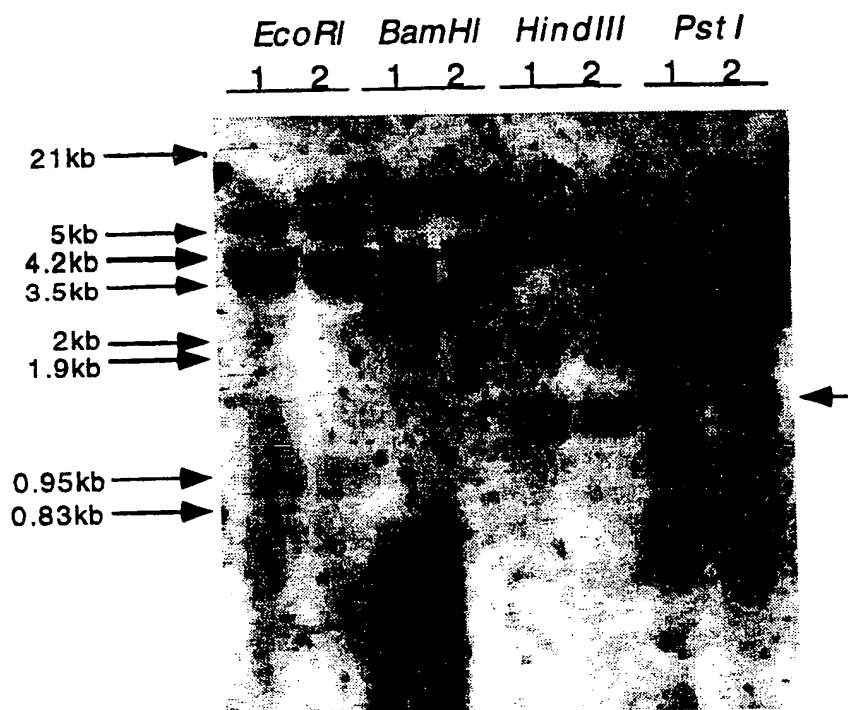
FIGURE 2

**FIGURE 3**

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**FIGURE 4**

SUBSTITUTE SHEET (Rule 26) (RO/AU)

**FIGURE 5**

SUBSTITUTE SHEET (Rule 26) (RO/AU)

mu.hep	0
rat.hep	0
hu.hep	MLLRSKPALP	PPLMLLLLGP	LGPLSPGALP	30
mu.hep	0
rat.hep	0
hu.hep	RPAQAQDVVD	LDFFTQEPLH	LVSPSFLSVT	60
mu.hep	0
rat.hep	0
hu.hep	IDANLATDPR	FLILLGSPKL	RTLARGLSPA	90
mu.hep	0
rat.hep	0
hu.hep	YLRFGGTKTD	FLIFDPKKES	TFEERSYWQS	120
mu.hep	0
rat.hep	0
hu.hep	QVNQDICKYG	SIPPDVEEKL	RLEWPYQEQL	150
mu.hepTYSRSSV	DMLYSFAKCS	17
rat.hepTYSRSSV	DMLYSFAKCS	17
hu.hep	LLREHYQKKF	KNSTYSRSSV	DVLYTFANCS	180
mu.hep	GLDLIFGLNA	LLGTPDLRWN	SSNAQLLLDY	47
rat.hep	RLDLIFGLNA	LLRTPDLRWN	SSNAQLLLNY	47
hu.hep	GLDLIFGLNA	LLRTADLQWN	SSNAQLLLDY	210

FIGURE 6-1

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mu.hep	CSSKGYNISW	ELGNEPNSFW	KKAHILIDGL	77
rat.hep	CSSKGYNICW	ELGNEPNSFW	KKAHISIDGL	77
hu.hep	CSSKGYNISW	ELGNEPNSFL	KKADIFINGS	240
mu.hep	QLGEDFVELH	KLLQRSASFQ	AKLYGPDIGQ	107
rat.hep	QLGEDFVELH	KLLQPSASFQ	AKLYGPDIGQ	107
hu.hep	QLGEDFIQLH	KLLRKSTFKN	AKLYGPDVGQ	270
mu.hep	PRGKTVKLLR	SFLKAGGEVI	DSLTHWHYYL	137
rat.hep	PRGKTVKLLR	SFLKAGGEVI	DSLTHWHYYL	137
hu.hep	PRRKTAKMLK	SFLKAGGEVI	DSVTHWHYYL	300
mu.hep	NGRIATKEDF	LSSDVLDTFI	LSVQKILKVT	167
rat.hep	NGRVATKEDF	LSSDVLDTFI	LSVQKILKVT	167
hu.hep	NGRTATREDF	LNPDVLDIFI	SSVQKVFQVV	330
mu.hep	KEITPGKKVW	LGETSSAYGG	GAPLLSNTFA	197
rat.hep	KQMTPGKKVW	LGETSSAYGG	GAPLLSDTFA	197
hu.hep	ESTRPGKKVW	LGETSSAYGG	GAPLLSDTFA	360
mu.hep	AGFMWLDKLG	LSARMGIEVV	MRQVFFGAGN	227
rat.hep	AGFMWLDKLG	LSAQLGIEVV	MRQVFFGAGN	227
hu.hep	AGFMWLDKLG	LSARMGIEVV	MRQVFFGAGN	390

FIGURE 6-2

mu.hep	YHLVDENFEP	LPDYWLSLLF	KKLVGPRVLL	257
rat.hep	YHLVDENFEP	LPDYWLSLLF	KKLVGPKVLM	257
hu.hep	YHLVDENFDP	LPDYWLSLLF	KKLVGTKVLM	420
mu.hep	SRVKGPDORSK	LRVYLHCTNV	YHPRYQEGDL	287
rat.hep	SRVKGPDORSK	LRVYLHCTNV	YHPRYREGDL	287
hu.hep	ASVQGSKRK	LRVYLHCTNT	DNPRYKEGDL	450
mu.hep	TLYVLNLHNV	TKHLKVPPPL	FRKPVDTYLL	317
rat.hep	TLYVLNLHNV	TKHLKLPPPM	FSRPVDKYLL	317
hu.hep	TLYAINLHNV	TKYLRLPYPF	SNKQVDKYLL	480
mu.hep	KPSGPDGLLS	KSVQLNGQIL	KMVDEQTLPA	347
rat.hep	KPFGSDGLLS	KSVQLNGQTL	KMVDEQTLPA	347
hu.hep	RPLGPHGLLS	KSVQLNGLTL	KMVDDQTLPP	510
mu.hep	LTEKPLPAGS	ALSLPAFSYG	FFVIRNAKIA	377
rat.hep	LTEKPLPAGS	SLSVPAFSYG	FFVIRNAKIA	377
hu.hep	LMEKPLRPGS	SLGLPAFSYS	FFVIRNAKVA	540
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rat.hep	ACI	380		
hu.hep	ACI	543		

FIGURE 6-3

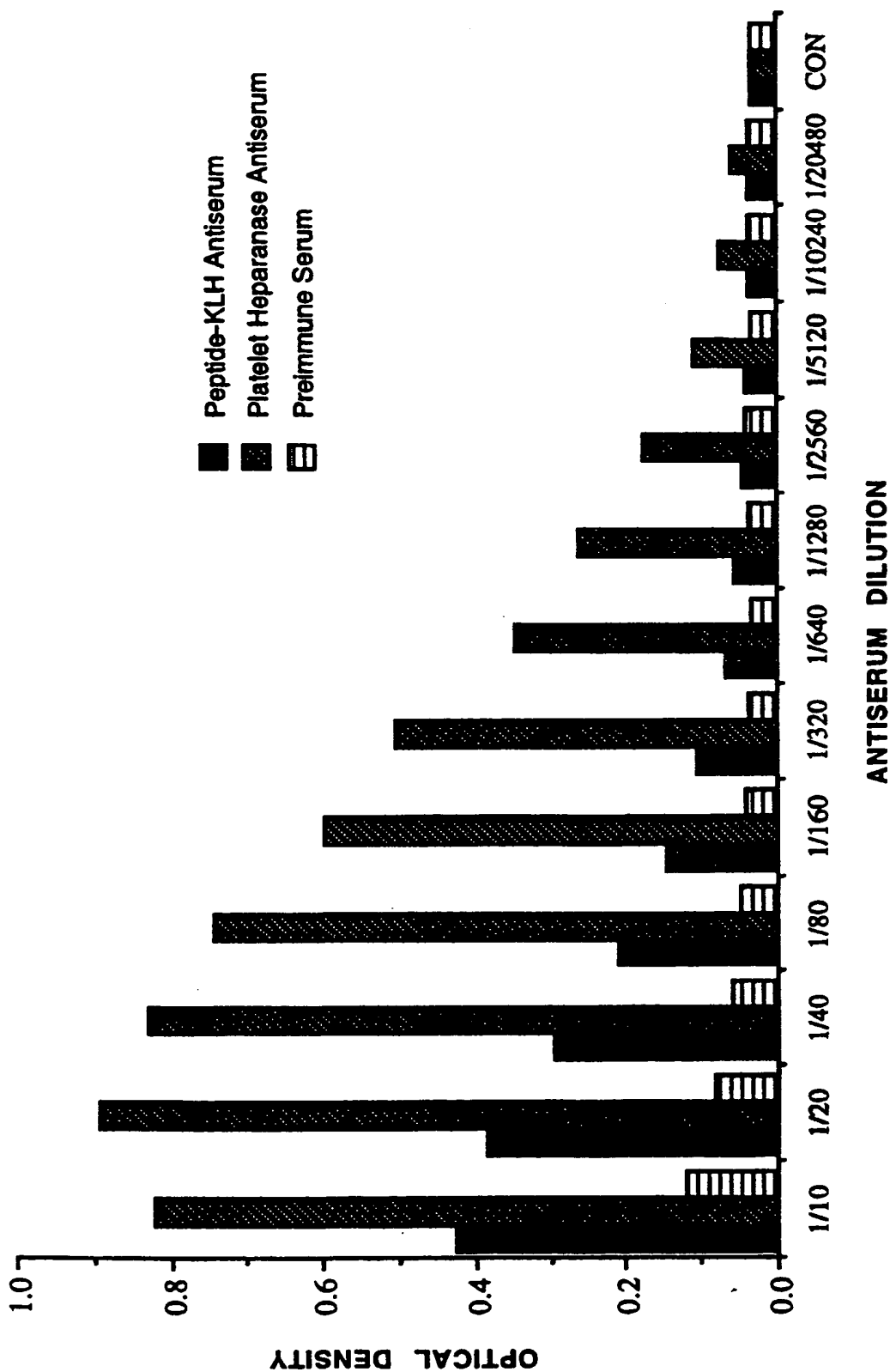


Figure 7

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU 98/00898

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl⁶: C12N 9/88, A61K 038/51

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
see electronic Database Box below

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
see electronic Database Box below

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Genebank, Embl, Swissprot - all sequences as claimed
WPAT- human platelet heparanase, glucuronidase, mammal, human
Medline - inventor name search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TURNBULL, J.E. and GALLAGHER, J.T., "Molecular Organization of heparan sulphate from human skin fibroblasts: Biochem. J., vol. 265, pages 715-724 cited in the application.	
A	TURNBULL, J.E. and GALLAGHER, J.T., (1991) "Distribution of iduronate 2-sulphate residues in heparan sulphate: Biochem. J., vol. 273, pages 553-559 cited in the application.	

☐ Further documents are listed in the continuation of Box C

☐ See patent family annex

<p>* Special categories of cited documents:</p>		
"A"	document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier application or patent but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search
13 November 1998

Date of mailing of the international search report
24 NOV 1998

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